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<p>(21) International Application Number: PCT/US95/13231</p> <p>(22) International Filing Date: 17 October 1995 (17.10.95)</p> <p>(30) Priority Data:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">08/326,110</td> <td style="width: 30%;">18 October 1994 (18.10.94)</td> <td style="width: 40%;">US</td> </tr> <tr> <td>08/461,965</td> <td>5 June 1995 (05.06.95)</td> <td>US</td> </tr> <tr> <td>08/465,380</td> <td>5 June 1995 (05.06.95)</td> <td>US</td> </tr> <tr> <td>08/486,397</td> <td>5 June 1995 (05.06.95)</td> <td>US</td> </tr> <tr> <td>08/486,399</td> <td>5 June 1995 (05.06.95)</td> <td>US</td> </tr> </table> <p>(71) Applicant (for all designated States except US): CORVAS INTERNATIONAL, INC. [US/US]; 3030 Science Park Road, San Diego, CA 92121 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): VLASUK, George, Phillip [US/US]; 3024 Garboso Street, Carlsbad, CA 92009 (US). STANSSENS, Patrick, Eric, Hugo [BE/BE]; Constant Permekelaan 48, B-9830 Saint-Martens-Latem (BE). MESSENS, Joris, Hilda, Lieven [BE/BE]; Desguinlei 234, B-2018 Antwerpen (BE). LAUWEREYS, Marc, Josef [BE/BE]; Wilgenstraat 2, B-9450 Haaltert (BE). LAROCHE, Yves, Rene [BE/BE]; Rue Bemel 115, B-1150 Brussels (BE). JESPER, Laurent, Stephane [US/US]; Karel van Lorrainenlaan 4, B-3080 Tervuren</p>			08/326,110	18 October 1994 (18.10.94)	US	08/461,965	5 June 1995 (05.06.95)	US	08/465,380	5 June 1995 (05.06.95)	US	08/486,397	5 June 1995 (05.06.95)	US	08/486,399	5 June 1995 (05.06.95)	US
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<p>(54) Title: NEMATODE-EXTRACTED SERINE PROTEASE INHIBITORS AND ANTICOAGULANT PROTEINS</p> <p>(57) Abstract</p> <p>Proteins which have activity as anticoagulants and/or serine protease inhibitors and have at least one NAP domain are described. Certain of these proteins have factor Xa inhibitory activity and others have activity as inhibitors of factor VIIa/TF. These proteins can be isolated from natural sources as nematodes, chemically synthesized or made by recombinant methods using various DNA expression systems.</p>																	
<pre> 1 10 20 30 G AATTCGCGCTA CTACTCAACA ATG AAG ATG CTT TAC OCT ATC OCT Met Lys Met Leu Tyr Ala Ile Ala 40 50 60 70 ATA ATG TTT CTC CTG GTA TCA TTA TGC AGC GCA AGA ACA GTG Ile Met Phe Leu Leu Val Ser Leu Cys Ser Ala Arg Thr Val 80 90 100 110 120 AGG AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp 130 140 150 160 GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG Asp Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys Cys Asn Glu 170 180 190 200 GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CAC TCA GGT GGT Glu Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg Ser Arg Gly 210 220 230 240 TOT TTA TTA CCT CCT CCT TGC GTA TGC AAA GAC GGA TTC TAC Cys Leu Leu Pro Pro Ala Cys Val Cys Lys Asp Gly Phe Tyr 250 260 270 280 AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC Arg Asp Thr Val Ile Gly Asp Cys Val Arg Glu Glu Glu Cys 290 300 310 320 330 GAC CAA CAT GAG ATT ATA CAT GTC TGA ACGAGAAAGC ACAAATAAC Asp Gln His Glu Ile Ile His Val 340 350 360 370 380 AAAGGTTGCA ACTCTGCTC TCAGAAATCG CTAGTTGGAT GTCTCTTTG 390 400 410 420 430 CCTCCGAATA GTTTTAGTTG ATGTTAAGTA AGAACTCTGT CTGAGAGAA 440 450 TAAAGCTTC CAATCC poly(A) </pre>																	

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5 NEMATODE-EXTRACTED SERINE PROTEASE
INHIBITORS AND ANTICOAGULANT PROTEINS

Cross Reference to Related Application

This application is a Continuation-in-Part of United
10 States Serial Nos. 08/461,965, 08/465,380, 08/486,397 and
08/486,399, all filed on June 5, 1995, each of which is a
continuation-in-part of U.S.S.N. 08/326,110, filed October
18, 1995; the disclosures of all these applications are
incorporated herein by reference.

15

Field of the Invention

The present invention relates to specific proteins as
well as recombinant versions of these proteins which are
serine protease inhibitors, including potent
20 anticoagulants in human plasma. These proteins include
certain proteins extracted from nematodes. In another
aspect, the present invention relates to compositions
comprising these proteins, which are useful as potent and
specific inhibitors of blood coagulation enzymes *in vitro*
25 and *in vivo*, and methods for their use as *in vitro*
diagnostic agents, or as *in vivo* therapeutic agents, to
prevent the clotting of blood. In a further aspect, the
invention relates to nucleic acid sequences, including
mRNA and DNA, encoding the proteins and their use in
30 vectors to transfect or transform host cells and as probes
to isolate certain related genes in other species and
organisms.

5 Background and Introduction to the Invention

Normal hemostasis is the result of a delicate balance between the processes of clot formation (blood coagulation) and clot dissolution (fibrinolysis). The complex interactions between blood cells, specific plasma proteins
10 and the vascular surface, maintain the fluidity of blood unless injury occurs. Damage to the endothelial barrier lining the vascular wall exposes underlying tissue to these blood components. This in turn triggers a series of
15 biochemical reactions altering the hemostatic balance in favor of blood coagulation which can either result in the desired formation of a hemostatic plug stemming the loss of blood or the undesirable formation of an occlusive intravascular thrombus resulting in reduced or complete lack of blood flow to the affected organ.

20 The blood coagulation response is the culmination of a series of amplified reactions in which several specific zymogens of serine proteases in plasma are activated by limited proteolysis. This series of reactions results in the formation of an insoluble matrix composed of fibrin and
25 cellular components which is required for the stabilization of the primary hemostatic plug or thrombus. The initiation and propagation of the proteolytic activation reactions occurs through a series of amplified pathways which are localized to membranous surfaces at the site of vascular
30 injury (Mann, K.G., Nesheim, M.E., Church, W.R., Haley, P. and Krishnaswamy, S. (1990) *Blood* 76: 1-16. and Lawson, J.H., Kalafatis, M., Stram, S., and Mann, K.G. (1994) *J. Biol. Chem.* 269: 23357-23366).

Initiation of the blood coagulation response to
35 vascular injury follows the formation of a catalytic complex composed of serine protease factor VIIa and the non-enzymatic co-factor, tissue factor (TF) (Rappaport, S.I. and Rao, L.V.M. (1992) *Arteriosclerosis and Thrombosis* 12: 1112-1121). This response appears to be exclusively
40 regulated by the exposure of subendothelial TF to trace circulating levels of factor VIIa and its zymogen factor VII, following a focal breakdown in vascular integrity.

- 5 Autoactivation results in an increase in the number of factor VIIa/TF complexes which are responsible for the formation of the serine protease factor Xa. It is believed that in addition to the factor VIIa/TF complex, the small amount of factor Xa which is formed primes the coagulation response through the proteolytic modification of factor IX to factor IX_{alpha} which in turn is converted to the active serine protease factor IX_{beta} by the factor VIIa/TF complex (Mann, K.G., Krishnaswamy, S. and Lawson, J.H. (1992) *Sem. Hematology* 29: 213-226.). It is factor IX_{beta} in complex with activated factor VIIIa, which appears to be responsible for the production of significant quantities of factor Xa which subsequently catalyzes the penultimate step in the blood coagulation cascade; the formation of the serine protease thrombin.
- 20 Factor Xa catalyzes the formation of thrombin following the assembly of the prothrombinase complex which is composed of factor Xa, the non-enzymatic co-factor Va and the substrate prothrombin (factor II) assembled in most cases, on the surface of activated platelets which are adhered at the site of injury (Fuster, V., Badimon, L., Badimon, J.J. and Chesebro, J.H. (1992) *New Engl. J. Med.* 326: 310-318). In the arterial vasculature, the resulting amplified "burst" of thrombin generation catalyzed by prothrombinase causes a high level of this protease locally which is responsible for the formation of fibrin and the further recruitment of additional platelets as well as the covalent stabilization of the clot through the activation of the transglutaminase zymogen factor XIII. In addition, the coagulation response is further propagated through the thrombin-mediated proteolytic feedback activation of the non-enzymatic co-factors V and VIII resulting in more prothrombinase formation and subsequent thrombin generation (Hemker, H.C. and Kessels, H. (1991) *Haemostasis* 21: 189-196).
- 40 Substances which interfere in the process of blood coagulation (anticoagulants) have been demonstrated to be important therapeutic agents in the treatment and

5 prevention of thrombotic disorders (Kessler, C.M. (1991)
Chest 99: 97S-112S and Cairns, J.A., Hirsh, J., Lewis,
H.D., Resnekov, L., and Theroux, P. (1992) Chest 102: 456S-
481S). The currently approved clinical anticoagulants have
10 been associated with a number of adverse effects owing to
the relatively non-specific nature of their effects on the
blood coagulation cascade (Levine, M.N., Hirsh, J.,
Landefeld, S., and Raskob, G. (1992) Chest 102: 352S-363S).
This has stimulated the search for more effective
15 anticoagulant agents which can more effectively control the
activity of the coagulation cascade by selectively
interfering with specific reactions in this process which
may have a positive effect in reducing the complications of
anticoagulant therapy (Weitz, J., and Hirsh, J. (1993) J.
Lab. Clin. Med. 122: 364-373). In another aspect, this
20 search has focused on normal human proteins which serve as
endogenous anticoagulants in controlling the activity of
the blood coagulation cascade. In addition, various
hematophageous organisms have been investigated because of
their ability to effectively anticoagulate the blood meal
25 during and following feeding on their hosts suggesting that
they have evolved effective anticoagulant strategies which
may be useful as therapeutic agents.

A plasma protein, Tissue Factor Pathway Inhibitor
(TFPI), contains three consecutive Kunitz domains and has
30 been reported to inhibit the enzyme activity of factor Xa
directly and, in a factor Xa-dependent manner, inhibit the
enzyme activity of the factor VIIa-tissue factor complex.
Salvensen, G., and Pizzo, S.V., "Proteinase Inhibitors: α -
Macroglobulins, Serpins, and Kunis", "Hemostasis and
35 Thrombosis, Third Edition, pp. 251-253, J.B. Lippincott
Company (Edit. R.W. Colman et al. 1994). A cDNA sequence
encoding TFPI has been reported, and the cloned protein was
reported to have a molecular weight of 31,950 daltons and
contain 276 amino acids. Broze, G.J. and Girad, T.J., U.S.
40 Patent No. 5,106,833, col. 1, (1992). Various recombinant
proteins derived from TFPI have been reported. Girad, T.J.
and Broze, G.J., EP 439,442 (1991); Rasmussen, J.S. and

- 5 Nordfand, O.J., WO 91/02753 (1991); and Broze, G.J. and Girad, T.J., U.S. Patent No. 5,106,833, col. 1, (1992).

Antistasin, a protein comprised of 119 amino acids and found in the salivary gland of the Mexican leech, *Haementeria officinalis*, has been reported to inhibit the
10 enzyme activity of factor Xa. Tuszynski et al., J. Biol. Chem, 262:9718 (1987); Nutt, et al., J. Biol. Chem, 263:10162 (1988). A 6,000 daltons recombinant protein containing 58 amino acids with a high degree homology to antistasin's amino-terminus amino acids 1 through 58 has
15 been reported to inhibit the enzyme activity of factor Xa. Tung, J. et al., EP 454,372 (October 30, 1991); Tung, J. et al., U.S. Patent No. 5,189,019 (February 23, 1993).

Tick Anticoagulant Peptide (TAP), a protein comprised of 60 amino acids and isolated from the soft tick,
20 *Ornithodoros moubata*, has been reported to inhibit the enzyme activity of factor Xa but not factor VIIa. Waxman, L. et al., Science, 248:593 (1990). TAP made by recombinant methods has been reported. Vlausk, G.P. et al., EP 419,099 (1991) and Vlausk, G.P. et al., U.S. Patent No
25 5,239,058 (1993).

The dog hookworm, *Ancylostoma caninum*, which can also infect humans, has been reported to contain a potent anticoagulant substance which inhibited coagulation of blood in vitro. Loeb, L. and Smith, A.J., Proc. Pathol.
30 Soc. Philadelphia, 7:173-187 (1904). Extracts of *A. caninum* were reported to prolong prothrombin time and partial thromboplastin time in human plasma with the anticoagulant effect being reported attributable to inhibition of factor Xa but not thrombin. Spellman, Jr.,
35 J.J. and Nossel, H.L., Am. J. Physiol., 220:922-927 (1971). More recently, soluble protein extracts of *A. caninum* were reported to prolong prothrombin time and partial thromboplastin time in human plasma in vitro. The anticoagulant effect was reported to be attributable to
40 inhibition of human factor Xa but not thrombin, Cappello, M, et al., J. Infect. Diseases, 167:1474-1477 (1993), and

5 to inhibition of factor Xa and factor VIIa (WO94/25000;
U.S. Patent No. 5,427,937).

The human hookworm, *Ancylostoma ceylanicum*, has also
been reported to contain an anticoagulant. Extracts of *A.*
ceylanicum have been reported to prolong prothrombin time
10 and partial thromboplastin time in dog and human plasma *in*
vitro. Carroll, S.M., et al., *Thromb. Haemostas.*
(Stuttgart), 51:222-227 (1984).

Soluble extracts of the non-hematophagous parasite,
Ascaris suum, have been reported to contain an
15 anticoagulant. These extracts were reported to prolong
the clotting of whole blood, as well as clotting time in
the kaolin-activated partial thromboplastin time test but
not in the prothrombin time test. Crawford, G.P.M. et al.,
J. Parasitol., 68: 1044-1047 (1982).

20 Chymotrypsin/elastase inhibitor-1 and its major isoforms,
trypsin inhibitor-1 and chymotrypsin/elastase inhibitor-4,
isolated from *Ascaris suum*, were reported to be serine
protease inhibitors and share a common pattern of five-
disulfide bridges. Bernard, V.D. and Peanasky, R.J., *Arch.*
25 *Biochem. Biophys.*, 303:367-376 (1993); Huang, K. et al.,
Structure, 2:679-689 (1994); and Grasberger, B.L. et al.,
Structure, 2:669-678 (1994). There was no indication that
the reported serine protease inhibitors had anticoagulant
activity.

30 Secretions of the hookworm *Necator americanus* are
reported to prolong human plasma clotting times, inhibit
the amidolytic activity of human FXa using a fluorogenic
substrate, inhibit multiple agonist-induced platelet dense
granule release, and degrade fibrinogen. Pritchard, D.I.
35 and B. Furmidge, *Thromb. Haemost.* 73: 546 (1995)
(WO95/12615).

Summary of the Invention

The present invention is directed to isolated
40 proteins having serine protease inhibiting activity and/or
anticoagulant activity and including at least one NAP
domain. We refer to these proteins as Nematode-extracted

5 Anticoagulant Proteins or "NAPs". "NAP domain" refers to a sequence of the isolated protein, or NAP, believed to have the inhibitory activity, as further defined herein below. The anticoagulant activity of these proteins may be assessed by their activities in increasing clotting
10 time of human plasma in the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays, as well as by their ability to inhibit the blood coagulation enzymes factor Xa or factor VIIa/TF. It is believed that the NAP domain is responsible for the observed
15 anticoagulant activity of these proteins. Certain of these proteins have at least one NAP domain which is an amino acid sequence containing less than about 120 amino acid residues, and including 10 cysteine amino acid residues.

20 In another aspect, the present invention is directed to a method of preparing and isolating a cDNA molecule encoding a protein exhibiting anticoagulant activity and having a NAP domain, and to a recombinant cDNA molecule made by this method. This method comprises the steps of:
25 (a) constructing a cDNA library from a species of nematode; (b) ligating said cDNA library into an appropriate cloning vector; (c) introducing said cloning vector containing said cDNA library into an appropriate host cell; (d) contacting the cDNA molecules of said host
30 cell with a solution containing a hybridization probe having a nucleic acid sequence comprising AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG, [SEQ. ID. NO. 94] wherein R is A or G, Y is T or C, and i is inosine; (e) detecting a recombinant cDNA molecule which hybridizes to said probe;
35 and (f) isolating said recombinant cDNA molecule.

In another aspect, the present invention is directed to a method of making a recombinant protein encoded by said cDNA which has anticoagulant activity and which includes a NAP domain and to recombinant proteins made by
40 this method. This method comprises the steps of: (a) constructing a cDNA library from a species of nematode; (b) ligating said cDNA library into an appropriate cloning

5 vector; (c) introducing said cloning vector containing
said cDNA library into an appropriate host cell; (d)
contacting the cDNA molecules of said host cell with a
solution containing a hybridization probe having a nucleic
acid sequence comprising AAR GCi TAY Cci GAR TGY GGi GAR
10 AAY GAR TGG, wherein R is A or G, Y is T or C, and i is
inosine [SEQ. ID. NO. 94]; (e) detecting a recombinant
cDNA molecule which hybridizes to said probe; (f)
isolating said recombinant cDNA molecule; (g) ligating the
nucleic acid sequence of said cDNA molecule which encodes
15 said recombinant protein into an appropriate expression
cloning vector; (h) transforming a second host cell with
said expression cloning vector containing said nucleic
acid sequence of said cDNA molecule which encodes said
recombinant protein; (i) culturing the transformed second
20 host cell; and (j) isolating said recombinant protein
expressed by said second host cell. It is noted that when
describing production of recombinant proteins in certain
expression systems such as COS cells, the term
"transfection" is conventionally used in place of (and
25 sometimes interchangeably with) "transformation".

In another aspect, the present invention is directed
to a method of making a recombinant cDNA encoding a
recombinant protein having anticoagulant activity and
having a NAP domain, comprising the steps of: (a)
30 isolating a cDNA library from a nematode;
(b) ligating said cDNA library into a cloning vector;
(c) introducing said cloning vector containing said cDNA
library into a host cell; (d) contacting the cDNA
molecules of said host cells with a solution comprising
35 first and second hybridization probes, wherein said first
hybridization probe has the nucleic acid sequence
comprising AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC
GAC GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT
GAG GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC TCA CGT
40 GGT TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC
TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA
TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 1],

- 5 and said second hybridization probe has the nucleic acid sequence comprising AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC AAG TGC AGT GAG GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA
10 TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 2];
- (e) detecting a recombinant cDNA molecule which hybridizes to said mixture of said probes; and (f) isolating said
15 recombinant cDNA molecule.

In yet another aspect, the present invention is directed to a method of making a recombinant cDNA encoding a protein having anticoagulant activity and which encodes a NAP domain, comprising the steps of: (a) isolating a
20 cDNA library from a nematode; (b) ligating said cDNA library into an appropriate phagemid expression cloning vector; (c) transforming host cells with said vector containing said cDNA library; (d) culturing said host cells; (e) infecting said host cells with a helper phage;
25 (f) separating phage containing said cDNA library from said host cells; (g) combining a solution of said phage containing said cDNA library with a solution of biotinylated human factor Xa; (h) contacting a streptavidin-coated solid phase with said solution
30 containing said phages containing said cDNA library, and said biotinylated human factor Xa; (i) isolating phages which bind to said streptavidin-coated solid phase; and (j) isolating the recombinant cDNA molecule from phages which bind to said streptavidin-coated solid phase.

35 In one preferred aspect, the present invention is directed to a recombinant cDNA having a nucleic acid sequence selected from the nucleic acid sequences depicted in Figure 1, Figure 3, Figures 7A to 7F, Figure 9, Figures 13A to 13H, and Figure 14.

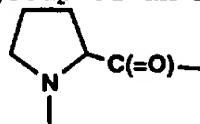
40 The present invention also is directed to NAPs that inhibit the catalytic activity of FXa, to NAPs that inhibit the catalytic activity of the FVIIa/TF complex,

5 and to NAPs that inhibit the catalytic activity of a serine protease, as well as nucleic acids encoding such NAPs and their methods of use.

Definitions.

10 The term "amino acid" refers to the natural L-amino acids; D-amino acids are included to the extent that a protein including such D-amino acids retains biological activity. Natural L-amino acids include alanine (Ala),
15 arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val).

20 The term "amino acid residue" refers to radicals having the structure: (1) -NH-CH(R)C(=O)- , wherein R is the alpha-carbon side-chain group of an L-amino acid,



except for L-proline; or (2) for L-proline.

The term "peptide" refers to a sequence of amino
25 acids linked together through their alpha-amino and carboxylate groups by peptide bonds. Such sequences as shown herein are presented in the amino to carboxy direction, from left to right.

The term "protein" refers to a molecule comprised of
30 one or more peptides.

The term "cDNA" refers to complementary DNA.

The term "nucleic acid" refers to polymers in which
bases (e.g., purines or pyrimidines) are attached to a
sugar phosphate backbone. Nucleic acids include DNA and
35 RNA.

The term "nucleic acid sequence" refers to the
sequence of nucleosides comprising a nucleic acid. Such
sequences as shown herein are presented in the 5' to 3'
direction, from left to right.

5 The term "recombinant DNA molecule" refers to a DNA molecule created by ligating together pieces of DNA that are not normally contiguous.

 The term "mRNA" refers to messenger ribonucleic acid.

 The term "homology" refers to the degree of
10 similarity of DNA or peptide sequences.

 The terms "Factor Xa" or "fXa" or "FXa" are synonymous and are commonly known to mean a serine protease within the blood coagulation cascade of enzymes that functions as part of the prothrombinase complex to
15 form the enzyme thrombin.

 The phrase "Factor Xa inhibitory activity" means an activity that inhibits the catalytic activity of fXa toward its substrate.

 The phrase "Factor Xa selective inhibitory activity"
20 means inhibitory activity that is selective toward Factor Xa compared to other related enzymes, such as other serine proteases.

 The phrase "Factor Xa inhibitor" is a compound having Factor Xa inhibitory activity.

25 The terms "Factor VIIa/Tissue Factor" or "fVIIa/TF" or "FVIIa/TF" are synonymous and are commonly known to mean a catalytically active complex of the serine protease coagulation factor VIIa (fVIIa) and the non-enzymatic protein Tissue Factor (TF), wherein the complex is
30 assembled on the surface of a phospholipid membrane of defined composition.

 The phrase "fVIIa/TF inhibitory activity" means an activity that inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically
35 inactive fXa derivative.

 The phrase "fVIIa/TF selective inhibitory activity" means fVIIa/TF inhibitory activity that is selective toward fVIIa/TF compared to other related enzymes, such as other serine proteases, including FVIIa and fXa.

40 The phrase a "fVIIa/TF inhibitor" is a compound having fVIIa/TF inhibitory activity in the presence of fXa or catalytically inactive fXa derivatives.

5 The phrase "serine protease" is commonly known to mean an enzyme, comprising a triad of the amino acids histidine, aspartic acid and serine, that catalytically cleaves an amide bond, wherein the serine residue within the triad is involved in a covalent manner in the
10 catalytic cleavage. Serine proteases are rendered catalytically inactive by covalent modification of the serine residue within the catalytic triad by diisopropylfluorophosphate (DFP).

 The phrase "serine protease inhibitory activity"
15 means an activity that inhibits the catalytic activity of a serine protease.

 The phrase "serine protease selective inhibitory activity" means inhibitory activity that is selective toward one serine protease compared to other serine
20 proteases.

 The phrase "serine protease inhibitor" is a compound having serine protease inhibitory activity.

 The term "prothrombinase" is commonly known to mean a catalytically active complex of the serine protease
25 coagulation Factor Xa (fXa) and the non-enzymatic protein Factor Va (fVa), wherein the complex is assembled on the surface of a phospholipid membrane of defined composition.

 The phrase "anticoagulant activity" means an activity that inhibits the clotting of blood, which includes the
30 clotting of plasma.

 The term "selective", "selectivity", and permutations thereof, when referring to NAP activity toward a certain enzyme, mean the NAP inhibits the specified enzyme with at least 10-fold higher potency than it inhibits other,
35 related enzymes. Thus, the NAP activity is selective toward that specified enzyme.

 The term "substantially the same" when used to refer to proteins, amino acid sequences, cDNAs, nucleotide sequences and the like refers to proteins, cDNAs or
40 sequences having at least about 90% homology with the other protein, cDNA, or sequence.

5 The term "NAP" or "NAP protein" means an isolated protein which includes at least one NAP domain and having serine protease inhibitory activity and/or anticoagulant activity.

10 Brief Description of the Drawings.

 Figure 1 depicts the nucleotide sequence of the AcaNAP5 cDNA [SEQ. ID. NO. 3]. The numbering starts at the first nucleotide of the cDNA. Translation starts at the first ATG codon (position 14); a second in frame ATG
15 is present at position 20.

 Figure 2 depicts the amino acid sequence of mature AcaNAP5 [SEQ. ID. NO. 4].

 Figure 3 depicts the nucleotide sequence of the AcaNAP6 cDNA [SEQ. ID. NO. 5]. The numbering starts at
20 the first nucleotide of the cDNA. Translation starts at the first ATG codon (position 14); a second in frame ATG is present at position 20.

 Figure 4 depicts the amino acid sequence of mature AcaNAP6 [SEQ. ID. NO. 6]. Amino acids that differ from
25 AcaNAP5 are underlined. In addition to these amino acid substitutions, AcaNAP6 contains a two amino acid deletion (Pro-Pro) when compared to AcaNAP5.

 Figure 5 depicts the amino acid sequence of Pro-AcaNAP5 [SEQ. ID. NO. 7].

30 Figure 6 depicts the amino acid sequence of Pro-AcaNAP6 [SEQ. ID. NO. 8]. Amino acids that differ from Pro-AcaNAP5 are underlined. In addition to these amino acid substitutions, Pro-AcaNAP6 contains a two amino acid deletion (Pro-Pro) when compared to Pro-AcaNAP5.

35 Figures 7A through 7F depict the nucleotide sequences of the cDNAs and deduced amino acid sequences of certain NAP proteins isolated from *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, and *Heligmosomoides polygyrus*. Figure 7A depicts sequences for the recombinant cDNA
40 molecule, AceNAP4, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 9]. Figure 7B depicts sequences for the recombinant cDNA molecule, AceNAP5, isolated from

5 *Ancylostoma ceylanicum* [SEQ. ID. NO. 10]. Figure 7C depicts sequences for the recombinant cDNA molecule, AceNAP7, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 11]. Figure 7D depicts sequences for the recombinant cDNA molecule, AduNAP4, isolated from *Ancylostoma*
10 *duodenale* [SEQ. ID. NO. 12]. Figure 7E depicts sequences for the recombinant cDNA molecule, AduNAP7, isolated from *Ancylostoma duodenale* [SEQ. ID. NO. 13]. Figure 7F depicts sequences for the recombinant cDNA molecule, HponAP5, isolated from *Heligmosomoides polygyrus* [SEQ. ID. NO. 14]. The EcoRI site, corresponding to the 5'-end of the recombinant cDNA molecule, is indicated in all cases (underlined). Numbering of each sequence starts at this EcoRI site. AceNAP4 and AduNAP7, each encode a protein which has two NAP domains; all other clones in this Figure
20 code for a protein having a single NAP domain. The AduNAP4 cDNA clone is not full-length, i.e., the recombinant cDNA molecule lacks the 5'-terminal part of the coding region based on comparison with other isoforms.

Figures 8A through 8C depict the nucleotide sequence of the vectors, pDONG61 (Figure 8A) [SEQ. ID. NO. 15], pDONG62 (Figure 8B) [SEQ. ID. NO. 16], and pDONG63 (Figure 8C) [SEQ. ID. NO. 17]. The HindIII-BamHI fragment which is shown is located between the HindIII and BamHI sites of pUC119. The vectors allow the cloning of cDNAs, as SfiI-NotI fragments, in the three different reading frames
30 downstream of the filamentous phage gene 6. All relevant restriction sites are indicated. The AAA Lys-encoding triplet at position 373-375 is the last codon of gene 6. The gene 6 encoded protein is followed by a Gly-Gly-Gly-Ser-Gly-Gly [SEQ. ID. NO. 18] linker sequence.
35

Figure 9 depicts the nucleotide sequence of the recombinant cDNA molecule, AcaNAPc2 cDNA [SEQ. ID. NO. 19]. The EcoRI site, corresponding to the 5'-end of the cDNA, is indicated (underlined). Numbering starts at this
40 EcoRI site. The deduced amino acid sequence is also shown; the translational reading frame was determined by the gene 6 fusion partner. The AcaNAPc2 cDNA lacks a

5 portion of the 5'-terminal part of the coding region; the homology with AcaNAP5 and AcaNAP6 predicts that the first seven amino acid residues belong to the secretion signal.

Figures 10A and 10B depict the comparative effects of certain NAP proteins on the prothrombin time (PT)
10 measurement (Figure 10A) and the activated partial thromboplastin time (aPTT) (Figure 10B) of normal citrated human plasma. Solid circles, (*), represent Pro-AcaNAP5; open triangles, (Δ), represent AcaNAP5 (AcaNAP5* in Table 2); and open circles, (O), represent native AcaNAP5.

15 Figure 11 depicts the alignment of the amino acid sequences encoded by certain NAP cDNAs isolated from various nematodes. AcaNAP5 [SEQ. ID. NO. 20], AcaNAP6 [SEQ. ID. NO. 21], and AcaNAPc2 [SEQ. ID. NO. 128] were isolated from *Ancylostoma caninum*. AceNAP5 [SEQ. ID. NO.
20 22], AceNAP7 [SEQ. ID. NO. 23], and AceNAP4 (AceNAP4d1 [SEQ. ID. NO. 24] and AceNAP4d2 [SEQ. ID. NO. 25] were isolated from *Ancylostoma ceylanicum*. AduNAP4 [SEQ. ID. NO. 26] and AduNAP7 (AduNAP7d1 [SEQ. ID. NO. 27] and AduNAP7d2 [SEQ. ID. NO. 28]) were isolated from
25 *Ancylostoma duodenale*. HpoNAP5 [SEQ. ID. NO. 29] was isolated from *Heligmosomoides polygyrus*. The amino acid sequences shown in this figure are as given in Figures 1, 3, 7A through 7F, and 9. The sequences of mature AcaNAP5 [SEQ. ID. NO. 4] and AcaNAP6 [SEQ. ID. NO. 6] (see Figures
30 2 and 4) are characterized, in part, by ten cysteine residues (numbered one through ten and shown in bold). All of the amino acid sequences in this Figure contain at least one NAP domain. The AceNAP4 cDNA consists of two adjacent regions, named AceNAP4d1 [SEQ. ID. NO. 24] and
35 AceNAP4d2 [SEQ. ID. NO. 25], which encode a first (d1) and second (d2) NAP-domain; similarly, the AduNAP7 cDNA contains two adjacent regions, AduNAP7d1 [SEQ. ID. NO. 27] and AduNAP7d2 [SEQ. ID. NO. 28], encoding a first (d1) and second (d2) NAP-domain. The alignment of the amino acid
40 sequences of all NAP-domains is guided by the cysteines; dashes (---) were introduced at certain positions to maintain the cysteine alignment and indicate the absence

5 of an amino acid at that position. The carboxy-terminal residue of a cDNA encoded protein is followed by the word "end".

Figures 12A and 12B depict a map of the *P. pastoris* pYAM7SP8 expression/secretion vector (Figure 12A) and
10 sequences included in the vector (Figure 12B) [SEQ. ID. NO. 30]. As depicted in Figure 12A, this plasmid contains the following elements inserted between the methanol-induced AOX1 promoter (dark arrow in the 5'AOX untranslated region) and the AOX1 transcription
15 termination signal (3'T): a synthetic DNA fragment encoding the acid phosphatase secretion signal (S), a synthetic 19-amino acid pro sequence (P) ending with a Lys-Arg processing site for the KEX2 protease and a multicloning site. The *HIS4* gene which serves as a
20 selection marker in GS115 transformation was modified by site directed mutagenesis to eliminate the *Stu*I recognition sequence (*HIS4**). pBR322 sequences, including the *Bla* gene and origin (*ori*) for propagation in *E. coli* are represented by a single line. Figure 12B depicts the
25 following contiguous DNA sequences which are incorporated in pYAM7SP8: the acid phosphatase (*PHO1*) secretion signal sequence, pro sequence and multicloning site (MCS) sequence. The ATG start codon of the *PHO1* secretion signal is underlined.

30 Figures 13A through 13H depict the nucleotide sequences of the cDNAs and deduced amino acid sequences of certain NAP proteins isolated from *Ancylostoma caninum*. Figure 13A depicts sequences for the recombinant cDNA molecule AcaNAP23 [SEQ. ID. NO. 31]. Figure 13B depicts
35 sequences for the recombinant cDNA molecule AcaNAP24 [SEQ. ID. NO. 32]. Figure 13C depicts sequences for the recombinant cDNA molecule AcaNAP25 [SEQ. ID. NO. 33]. Figure 13D depicts sequences for the recombinant cDNA molecules AcaNAP31, AcaNAP42, and AcaNAP46, all of which
40 are identical [SEQ. ID. NO. 34]. Figure 13E depicts sequences for the recombinant cDNA molecule AcaNAP44 [SEQ. ID. NO. 35]. Figure 13F depicts sequences for the

5 recombinant cDNA molecule AcaNAP45 [SEQ. ID. NO. 36].
Figure 13G depicts sequences for the recombinant cDNA
molecule AcaNAP47 [SEQ. ID. NO. 37]. Figure 13H depicts
sequences for the recombinant cDNA molecule AcaNAP48 [SEQ.
ID. NO. 38]. The EcoRI site, corresponding to the 5'-end
10 of the recombinant cDNA molecule, is indicated in all
cases (underlined). Numbering of each sequence starts at
this EcoRI site. AcaNAP45 and AcaNAP47, each encode a
protein which has two NAP domains; all other clones in
this Figure code for a protein having a single NAP domain.
15 Figure 14 depicts the nucleotide, and deduced amino
acid, sequence of the recombinant cDNA molecule NamNAP
[SEQ. ID. NO. 39].

Figure 15 presents the antithrombotic activity of
AcaNAP5 and Low Molecular Weight Heparin (LMWH;
20 Enoxaparin™) evaluated in the FeCl₃ model of arterial
thrombosis. Activity data is represented as the percent
incidence of occlusive thrombus formation in the carotid
artery (circles). Thrombus formation began 150 minutes
after subcutaneous (s.c.) administration of test agent.
25 Deep wound bleeding was quantified in a separate group of
animals that were treated in an identical manner but
without addition of FeCl₃ (squares). Blood loss at a deep
surgical wound in the neck was quantified over a total of
210 minutes after subcutaneous compound administration.

30 Figure 16 presents the alignment of amino acid
sequences corresponding to mature NAPs isolated according
to the procedures disclosed herein: namely AcaNAP5 [SEQ.
ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID.
NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID.
35 NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID.
NO. 46], AcaNAP31, 42, 46 [SEQ. ID. NO. 47], AceNAP4d1
[SEQ. ID. NO. 48], AceNAP4d2 [SEQ. ID. NO. 49], AcaNAP45d1
[SEQ. ID. NO. 50], AcaNAP47d1 [SEQ. ID. NO. 51], AduNAP7d1
[SEQ. ID. NO. 52], AcaNAP45d2 [SEQ. ID. NO. 53],
40 AcaNAP47d2 [SEQ. ID. NO. 54], AduNAP4 [SEQ. ID. NO. 55],
AduNAP7d2 [SEQ. ID. NO. 56], AceNAP5 [SEQ. ID. NO. 57],
AceNAP7 [SEQ. ID. NO. 58], AcaNAPc2 [SEQ. ID. NO. 59],

5 HpoNAP5 [SEQ. ID. NO. 60], and NamNAP [SEQ. ID. NO. 61]. Each NAP domain comprises ten cysteine residues, which are used to align the sequences, and amino acid sequences between the cysteines. A1 through A10 represent the amino acid sequences between the cysteine residues.

10 Figure 17 depicts the amino acid sequence of mature AceNAP4 [SEQ. ID. NO. 62] having two NAP domains.

Figure 18 depicts the amino acid sequence of mature AcaNAP45 [SEQ. ID. NO. 63] having two NAP domains.

Figure 19 depicts the amino acid sequence of mature
15 AcaNAP47 [SEQ. ID. NO. 64] having two NAP domains.

Figure 20 depicts the amino acid sequence for mature AduNAP7 [SEQ. ID. NO. 65] having two NAP domains.

Detailed Description of the Invention.

20 This invention provides a family of proteins, collectively referred to as Nematode-extracted Anticoagulant Proteins (NAPs). These proteins are so designated because the first member originally isolated was extracted from a nematode, the canine hookworm,
25 *Ancylostoma caninum*. However, the designation NAP or NAP domain should not be considered to limit the proteins of the present invention by this or other natural source.

Individual NAP proteins are characterized by having at least one NAP domain and by having serine protease
30 inhibitory and/or anticoagulant activity. Such anticoagulant activity may be assessed by increases in clotting time in both the PT and aPTT assays described herein, by the inhibition of factor Xa or factor VIIa/TF activity, or by demonstration of activity in vivo.
35 Preferably, blood or plasma used in such assays derives from species known to be infected by nematodes, such as pigs, humans, primates, and the like. The NAP domain is an amino acid sequence. It is believed that the NAP domain is responsible for the observed inhibitory and/or
40 anticoagulant activity. Certain representative NAP domains include the amino acid sequences depicted in Figures 11 and 16, particularly the sequences between the

5 cysteines designated as Cysteine 1 and Cysteine 10 in the
Figures and the sequence following Cysteine 10. The
characteristics broadly defining this family of proteins,
as well as the nucleic acid molecules, including mRNAs
sequences and DNA sequences which encode such proteins,
10 are provided. Methods of making these proteins, as well
as methods of making nucleic acid molecules encoding such
proteins, are also provided. The specific examples
provided are exemplary only and other members of the NAP
family of proteins, as well as nucleic acid sequences
15 encoding them, can be obtained by following the procedures
outlined in these examples and described herein.

The proteins of the present invention include
isolated NAPs which comprise proteins having anticoagulant
activity and including at least one NAP domain.

20 With respect to "anticoagulant activity", the
purified proteins of the present invention are active as
anticoagulants, and as such, are characterized by
inhibiting the clotting of blood which includes the
clotting of plasma. In one aspect, the preferred isolated
25 proteins of the present invention include those which
increase the clotting time of human plasma as measured in
both the prothrombin time (PT) and activated partial
thromboplastin time (aPTT) assays.

In the PT assay, clotting is initiated by the
30 addition of a fixed amount of tissue factor-phospholipid
micelle complex (thromboplastin) to human plasma.
Anticoagulants interfere with certain interactions on the
surface of this complex and increase the time required to
achieve clotting relative to the clotting observed in the
35 absence of the anticoagulant. The measurement of PT is
particularly relevant for assessing NAP anticoagulant
activity because the series of specific biochemical events
required to cause clotting in this assay are similar to
those that must be overcome by the hookworm in nature to
40 facilitate feeding. Thus, the ability of NAP to act as an
inhibitor in this assay can parallel its activity in
nature, and is predictive of anticoagulant activity in

5 vivo. In both the assay and in nature, the coagulation response is initiated by the formation of a binary complex of the serine protease factor VIIa (fVIIa) and the protein tissue factor (TF) (fVIIa/TF), resulting in the generation of fXa. The subsequent assembly of fXa into the
10 prothrombinase complex is the key event responsible for the formation of thrombin and eventual clot formation.

In the aPTT assay, clotting is initiated by the addition of a certain fixed amount of negatively charged phospholipid micelle (activator) to the human plasma.
15 Substances acting as anticoagulants will interfere with certain interactions on the surface of the complex and again increase the time to achieve a certain amount of clotting relative to that observed in the absence of the anticoagulant. Example B describes such PT and aPTT
20 assays. These assays can be used to assess anticoagulant activity of the isolated NAPs of the present invention.

The preferred isolated NAPs of the present invention include those which double the clotting time of human plasma in the PT assay when present at a concentration of about 1 to about 500 nanomolar and which also double the
25 clotting time of human plasma in the aPTT assay when present at a concentration of about 1 to about 500 nanomolar. Especially preferred are those proteins which double the clotting time of human plasma in the PT assay
30 when present at a concentration of about 5 to about 100 nanomolar, and which also double the clotting time of human plasma in the aPTT assay when present at a concentration of about 5 to about 200 nanomolar. More especially preferred are those proteins which double the
35 clotting time of human plasma in the PT assay when present at a concentration about 10 to about 50 nanomolar, and which also double the clotting time of human plasma in the aPTT assay when present at a concentration of about 10 to about 100 nanomolar.

40 Anticoagulant, or antithrombotic, activity of NAPs of the present invention also can be evaluated using the in vivo models presented in Example F. The rat FeCl₃ model

5 described in part A of that Example is a model of platelet dependent, arterial thrombosis that is commonly used to assess antithrombotic compounds. The model evaluates the ability of a test compound to prevent the formation of an occlusive thrombus induced by FeCl_3 in a segment of the
10 rat carotid artery. NAPs of the present invention are effective anticoagulants in this model when administered intravenously or subcutaneously. The deep wound bleeding assay described in part B of Example F allows measurement of blood loss after administration of an anticoagulant
15 compound. A desired effect of an anticoagulant is that it inhibits blood coagulation, or thrombus formation, but not so much as to prevent clotting altogether and thereby potentiate bleeding. Thus, the deep wound bleeding assay measures the amount of blood loss over the 3.5 hour period
20 after administration of anticoagulant. The data presented in Figure 15 show NAP of the present invention to be an effective antithrombotic compound at a dose that does not cause excessive bleeding. In contrast, the dose of low molecular weight heparin (LMWH) that correlated with 0%
25 occlusion caused about three times more bleeding than the effective dose of NAP.

General NAP Domain [FORMULA I]

With respect to "NAP domain", the isolated proteins
30 (or NAPs) of the present invention include at least one NAP domain in their amino acid sequence. Certain NAP domains have an amino acid sequence having a molecular weight of about 5.0 to 10.0 kilodaltons, preferably from about 7.0 to 10.0 kilodaltons, and containing 10 cysteine
35 amino acid residues.

Certain preferred isolated NAPs of the present invention include those which contain at least one NAP domain, wherein each such NAP domain is further characterized by including the amino acid sequence: Cys-
40 A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys ("FORMULA I"),

5 wherein: (a) A₁ is an amino acid sequence containing 7 to
8 amino acid residues; (b) A₂ is an amino acid sequence
containing 2 to 5 amino acid residues; (c) A₃ is an amino
acid sequence containing 3 amino acid residues; (d) A₄ is
an amino acid sequence containing 6 to 17 amino acid
10 residues; (e) A₅ is an amino acid sequence containing 3 to
4 amino acid residues; (f) A₆ is an amino acid sequence
containing 3 to 5 amino acid residues; (g) A₇ is an amino
acid residue; (h) A₈ is an amino acid sequence containing
10 to 12 amino acid residues; and (i) A₉ is an amino acid
15 sequence containing 5 to 6 amino acid residues. Other
NAPs having slightly different NAP domains (See FORMULAS
II to V) are encompassed within the present invention.

Especially preferred NAP domains include those
wherein A₂ is an amino acid sequence containing 4 to 5
20 amino acid residues and A₄ is an amino acid sequence
containing 6 to 16 amino acid residues. More preferred
are NAP domains wherein: (a) A₁ has Glu as its fourth
amino acid residue; (b) A₂ has Gly as its first amino acid
residue; (c) A₈ has Gly as its third amino acid residue
25 and Arg as its sixth amino acid residue; and (d) A₉ has
Val as its first amino acid residue. More preferably, A₃
has Asp or Glu as its first amino acid residue and Lys or
Arg as its third amino acid residue and A₇ is Val or Gln.
Also, more preferably A₈ has Leu or Phe as its fourth
30 amino acid residue and Lys or Tyr as its fifth amino acid
residue. Also preferred are NAP domains where, when A₈
has 11 or 12 amino acid residues, Asp or Gly is its
penultimate amino acid residue, and, where when A₈ has 10
amino acids, Gly is its tenth amino acid residue. For
35 expression of recombinant protein in certain expression
systems, a recombinant NAP may additionally include an
amino acid sequence for an appropriate secretion signal.
Certain representative NAP domains include the sequences
depicted in Figure 11 and Figure 16, particularly the
40 sequences between (and including) the cysteines designated
as Cysteine 1 and Cysteine 10 and following Cysteine 10.

5 According to a preferred aspect, provided are NAPs which include at least one NAP domain of Formula I wherein the NAP domain includes the amino acid sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ.
10 ID. NOS. 66 and 129; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 130 to 133; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 134 to 145; (d) Cys-A5 is selected from SEQ. ID. NOS. 146 and 147; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 148 to 150; (f) Cys-A7-
15 Cys-A8 is selected from one of SEQ. ID. NOS. 151 to 153; and (g) Cys-A9-Cys is selected from SEQ. ID. NOS. 154 and 155. Also preferred are such proteins wherein Cys-A2-Cys is selected from SEQ. ID. NOS. 130 and 131 and A3-Cys-A4 is selected from one of SEQ. ID. NOS. 135 to 145. More
20 preferred are those proteins having NAP domains wherein SEQ. ID. NOS. 66 and 129 have Glu at location 5; SEQ. ID. NOS. 130 and 131 have Gly at location 2; SEQ. ID. NOS. 151 to 153 have Gly at location 6 and Arg at location 9; and SEQ. ID. NOS. 154 and 155 have Val at location 2. More
25 preferably SEQ. ID. NOS. 151 to 153 have Val or Glu at location 2, Leu or Phe at location 7 and/or Lys or Tyr at location 8. It is further preferred that SEQ. ID. NO. 151 has Asp or Gly at location 14; SEQ. ID. NO. 152 has Asp or Gly at location 13; and SEQ. ID. NO. 153 has Gly at
30 location 13.

Certain NAPs of the present invention demonstrate specificity toward inhibiting a particular component in the coagulation cascade, such as fXa or the fVIIa/TF complex. The specificity of a NAP's inhibitory activity
35 toward a component in the coagulation cascade can be evaluated using the protocol in Example D. There, the ability of a NAP to inhibit the activity of a variety of serine proteases involved in coagulation is measured and compared. The ability of a NAP to inhibit the fVIIa/TF
40 complex also can be assessed using the protocols in Example E, which measure the ability of a NAP to bind fXa in either an inhibitory or noninhibitory manner and to

5 inhibit FVIIa when complexed with TF. AcaNAP5 and AcaNAP6 are examples of proteins having NAP domains that specifically inhibit fXa. AcaNAPc2 is a protein having a NAP domain that demonstrates selective inhibition of the fVIIa/TF complex when fXa, or a catalytically active or
10 inactive derivative thereof, is present.

NAPs having anticoagulant activity, including NAPs having Factor Xa inhibitory activity (FORMULA II)

Thus, in one aspect NAPs of the present invention
15 also include an isolated protein having anticoagulant activity, including an isolated protein having Factor Xa inhibitory activity, and having one or more NAP domains, wherein each NAP domain includes the sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
20 Cys-A9-Cys-A10 ("FORMULA II"),
wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
 - (b) A2 is an amino acid sequence;
 - 25 (c) A3 is an amino acid sequence of 3 amino acid residues;
 - (d) A4 is an amino acid sequence;
 - (e) A5 is an amino acid sequence of 3 to 4 amino
30 acid residues;
 - (f) A6 is an amino acid sequence;
 - (g) A7 is an amino acid;
 - (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
 - 35 (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
 - (j) A10 is an amino acid sequence;
- wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid
40 residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid residues.

5 Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention.

10 NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] have one NAP domain and are preferred NAPs according to this aspect of
15 the invention.

 Preferred NAP proteins according to one embodiment of this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6
20 is an amino acid sequence of 3 to 5 amino acid residues, and A10 is an amino acid sequence of 5 to 25 amino acid residues.

 Thus, according to one preferred aspect, provided are isolated proteins having anticoagulant activity, including
25 isolated proteins having activity as Factor Xa inhibitors, having at least one NAP domain of formula II which includes the following sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ.
30 ID. NOS. 67 and 156; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 157 to 159; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 160 to 173; (d) Cys-A5 is selected from SEQ. ID. NOS. 174 and 175; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 176 to 178; (f) Cys-A7-
35 Cys-A8 is selected from SEQ. ID. NOS. 179 and 180; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 181 to 183; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 184 to 204.

 In another preferred embodiment of this aspect of the
40 invention, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3_a is selected from the group consisting

5 of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg. Especially preferred A3 sequences are selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and
10 Glu-Thr-Lys.

In an additional preferred embodiment of this aspect of the invention, A4 is an amino acid sequence having a net anionic charge.

According to this aspect of the invention, a
15 preferred A7 amino acid residue is Val or Ile.

Another preferred embodiment of this aspect of the invention is one in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

- 20 (a) A8_a is the first amino acid residue in A8,
(b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
(c) A8_c through A8_g are independently selected amino acid residues.

25 Preferably, A8_c is Gly, A8_d is selected from the group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. An especially preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO.
30 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

An additional preferred embodiment is one in which A10 includes an amino sequence selected from the group
35 consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

NAP proteins AcaNAP5 and AcaNAP6 include the amino
40 acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74] in A10, and are preferred NAPs according to this embodiment of the invention.

5 In one embodiment of this aspect of the invention, a preferred NAP molecule is one wherein

 (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;

 (b) A4 is an amino acid sequence having a net
10 anionic charge;

 (c) A7 is selected from the group consisting of Val and Ile;

 (d) A8 includes an amino acid sequence selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and

 (e) A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

 Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting
25 blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5
30 and AcaNAP6 have one NAP domain and are preferred NAPs according to this embodiment of the invention.

 In another preferred embodiment, a NAP molecule is one wherein

 (a) A3 is selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys;

 (b) A4 is an amino acid sequence having a net anionic charge;

 (c) A7 is Val or Ile;

40 (d) A8 includes an amino acid sequence selected from the group consisting of A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78], A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO.

5 79], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80], A8_a-
A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and A8_a-A8_b-
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82], wherein at least
one of A8_a and A8_b is Glu or Asp;

(e) A9 is an amino acid sequence of five amino acid
10 residues; and

(f) A10 includes an amino acid sequence selected
from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID.
NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-
Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr
15 [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising
NAP proteins according to this embodiment, and methods of
inhibiting blood coagulation comprising administering NAP
proteins according to this embodiment also are
contemplated by this invention. NAP proteins within this
20 embodiment of the invention have at least one NAP domain.
Preferred are NAPs having one or two NAP domains.
Preferred are proteins having at least one NAP domain that
is substantially the same as that of either AcaNAP5 [SEQ.
ID. NO. 40] or AcaNAP6 [SEQ. ID. NO. 41]. NAP proteins
25 AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID.
NOS. 6 and 41] have one NAP domain and are especially
preferred NAPs according to this embodiment of the
invention.

Preferred NAP proteins having anticoagulant activity,
30 including those having Factor Xa inhibitory activity,
according to all the embodiments recited above for this
aspect of the invention, can be derived from a nematode
species. A preferred nematode species is selected from the
group consisting of *Ancylostoma caninum*, *Ancylostoma*
35 *ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and
Heligomosomoides polygyrus. Particularly preferred are
NAP proteins AcaNAP5 and AcaNAP6 derived from *Ancylostoma*
caninum.

This aspect of the invention also contemplates
40 isolated recombinant cDNA molecules encoding a protein
having anticoagulant and/or Factor Xa inhibitory activity,
wherein the protein is defined according to each of the

5 embodiments recited above for isolated NAP protein having
anticoagulant and/or Factor Xa inhibitory activity.
Preferred cDNAs according to this aspect of the invention
code for AcaNAP5 and AcaNAP6.

10 The Factor Xa inhibitory activity of NAPs within this
aspect of the invention can be determined using protocols
described herein. Example A describes one such method.
In brief, a NAP is incubated with factor Xa for a period
of time, after which a factor Xa substrate is added. The
rate of substrate hydrolysis is measured, with a slower
15 rate compared to the rate in the absence of NAP indicative
of NAP inhibition of factor Xa. Example C provides
another method of detecting a NAP's inhibitory activity
toward factor Xa when it is assembled into the
prothrombinase complex, which more accurately reflects the
20 normal physiological function of fXa in vivo. As
described therein, factor Xa assembled in the
prothrombinase complex is incubated with NAP, followed by
addition of substrate. Factor Xa-mediated thrombin
generation by the prothrombinase complex is measured by
25 the rate of thrombin generation from this mixture.

NAPs having anticoagulant activity, including NAPs having
Factor VIIa/TF inhibitory activity (FORMULA III)

30 In another aspect, NAPs of the present invention also
include an isolated protein having anticoagulant activity,
including and isolated protein having Factor VIIa/TF
inhibitory activity and having one or more NAP domains,
wherein each NAP domain includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
35 Cys-A9-Cys-A10 ("FORMULA III"),

wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino
acid residues;
- (b) A2 is an amino acid sequence;
- 40 (c) A3 is an amino acid sequence of 3 amino acid
residues;
- (d) A4 is an amino acid sequence;

- 5 (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
- (f) A6 is an amino acid sequence;
- (g) A7 is an amino acid;
- 10 (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
- (j) A10 is an amino acid sequence;
- wherein each of A2, A4, A6 and A10 has an independently
15 selected number of independently selected amino acid residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid residues.

Pharmaceutical compositions comprising NAP proteins
20 according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are
25 NAPs having one or two NAP domains. Preferred are proteins having at least one NAP domain substantially the same as that of AcaNAPc2 [SEQ. ID. NO. 59]. NAP protein AcaNAPc2 [SEQ. ID. NO. 59] has one NAP domain and is an especially preferred NAP according to this aspect of the invention.

30 Preferred NAP proteins according to this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6 is an amino acid sequence of 3 to 5 amino acid residues, and A10
35 is an amino acid sequence of 5 to 25 amino acid residues.

Accordingly, in one preferred aspect, provided are NAPs having anticoagulant activity, including factor VIIa/TF inhibitory activity, and having at least one NAP domain of formula III wherein the NAP domain includes the
40 amino acid sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ.

5 ID. NOS. 83 and 205; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 206 to 208; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 209 to 222; (d) Cys-A5 is selected from SEQ. ID. NOS. 223 and 224; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 225 to 227; (f) Cys-A7-
10 Cys-A8 is selected from SEQ. ID. NOS. 228 and 229; (g) Cys-A9 is selected from SEQ. ID. NOS. 230 to 232; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 233 to 253.

In another preferred embodiment according to this aspect of the invention, A3 has the sequence Asp-A3_a-A3_b,
15 wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3 is Asp-Lys-Lys.

In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

In another preferred embodiment of this aspect of the
20 invention, A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 84], wherein A5_a through A5_d are independently selected amino acid residues. Preferably, A5_a is Leu and A5_c is Arg.

According to this aspect of the invention, a
25 preferred A7 amino acid residue is Val or Ile, more preferably Val.

An additional preferred embodiment of this aspect of the invention is one in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68],
30 wherein

(a) A8_a is the first amino acid residue in A8,
(b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
(c) A8_c through A8_g are independently selected amino
35 acid residues.

Preferably, A8_c is Gly, A8_d is selected from the group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. A preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is Gly-Phe-Tyr-Arg-Asn [SEQ.
40 ID. NO. 70].

In one embodiment, a preferred NAP molecule is one wherein:

- 5 (a) A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d, wherein A5_a through A5_d are independently selected amino acid residues; and
- 10 (d) A7 is selected from the group consisting of Val and Ile. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP protein AcaNAPc2 has one NAP domain and is a preferred NAP according to this embodiment of the invention.
- 15 20

In another preferred embodiment, a NAP molecule is one wherein

- (a) A3 is Asp-Lys-Lys;
- 25 (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 85], wherein A5_a through A5_d are independently selected amino acid residues;
- 30 (d) A7 is Val; and
- (e) A8 includes an amino acid sequence A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], wherein at least one of A8_a and A8_b is Glu or Asp. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP protein AcaNAPc2 [SEQ. ID. NO. 59] has one NAP domain and is a preferred NAP according to this embodiment of the invention.
- 35 40

5 Preferred NAP proteins having anticoagulant activity, including those having Factor VIIa/TF inhibitory activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the
10 group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*. Particularly preferred is NAP protein AcaNAPc2 derived from *Ancylostoma caninum*.

This aspect of the invention also contemplates
15 isolated recombinant cDNA molecules encoding a protein having anticoagulant and/or Factor VIIa/TF inhibitory activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having anticoagulant and/or Factor VIIa/TF inhibitory
20 activity. A preferred cDNA according to this aspect has a nucleotide sequence [SEQ. ID. NO. 19] and codes for AcaNAPc2 [SEQ. ID. NO. 59].

The fVIIa/TF inhibitory activity of NAPs within this aspect of the invention can be determined using protocols
25 described herein. Example E describes fVIIa/TF assays. There, the fVIIa/TF-mediated cleavage and liberation of the tritiated activation peptide from radiolabeled human factor IX (³H-FIX) or the amidolytic hydrolysis of a chromogenic peptidyl substrate are measured.
30 Interestingly, NAP fVIIa/TF inhibitors of the present invention require the presence of fXa in order to be active fVIIa/TF inhibitors. However, NAP fVIIa/TF inhibitors were equally effective in the presence of fXa in which the active site had been irreversibly occupied
35 with the peptidyl chloromethyl ketone H-Glu-Gly-Arg-CMK (EGR), and thereby rendered catalytically inactive (EGR-fXa). While not wishing to be bound by any one explanation, it appears that a NAP having fVIIa/TF inhibition activity forms a binary complex with fXa by
40 binding to a specific recognition site on the enzyme that is distinct from the primary recognition sites P₄-P₁, within the catalytic center of the enzyme. This is

5 followed by the formation of a quaternary inhibitory complex with the fVIIa/TF complex. Consistent with this hypothesis is that EGR-fXa can fully support the inhibition of fVIIa/TF by NAPs inhibitory for fVIIa/TF despite covalent occupancy of the primary recognition
10 sites (P4-P1) within the catalytic site of fXa by the tripeptidyl-chloromethyl ketone (EGR-CMK).

The fVIIa/TF inhibitory activity of NAPs also can be determined using the protocols in Example D, as well as the fXa assays described in Examples A and C. There, the
15 ability of a NAP to inhibit the catalytic activity of a variety of enzymes is measured and compared to its inhibitory activity toward the fVIIa/TF complex. Specific inhibition of fVIIa/TF by a NAP is a desired characteristic for certain applications.

20 A further aspect of the invention includes an isolated protein having anticoagulant activity, and cDNAs coding for the protein, wherein said protein specifically inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically inactive fXa
25 derivative, but does not specifically inhibit the activity of FVIIa in the absence of TF and does not specifically inhibit prothrombinase. Preferred proteins according to this aspect of the invention have the characteristics described above for an isolated protein having Factor
30 VIIa/TF inhibitory activity and having one or more NAP domains. A preferred protein according to this aspect of the invention is AcaNAPc2.

NAPs within this aspect of the invention are identified by their fVIIa/TF inhibitory activity in the
35 presence of fXa or a fXa derivative, whether the derivative is catalytically active or not. The protocols described in Examples B, C, and F are useful in determining the anticoagulant activity of such NAPs. The protocol in Example A can detect a NAP's inactivity toward
40 free fXa or prothrombinase. Data generated using the protocols in Example E will identify NAPs that require

5 either catalytically active or inactive fXa to inhibit fVIIa/TF complex.

NAPs having serine protease inhibitory activity (FORMULA IV)

10 In an additional aspect, NAPs of the present invention also include an isolated protein having serine protease inhibitory activity and having one or more NAP domains, wherein each NAP domain includes the sequence: Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
15 Cys-A9-Cys-A10, ("FORMULA IV") wherein
(a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
(b) A2 is an amino acid sequence;
(c) A3 is an amino acid sequence of 3 amino acid
20 residues;
(d) A4 is an amino acid sequence;
(e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
(f) A6 is an amino acid sequence;
25 (g) A7 is an amino acid;
(h) A8 is an amino acid sequence of 10 to 12 amino acid residues;
(i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
30 (j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid
35 residues. Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention. NAP proteins within this aspect of the
40 invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred are NAP domains that have amino acid sequences that are

5 substantially the same as the NAP domains of HpoNAP5 [SEQ. ID. NO. 60] or NamNAP [SEQ. ID. NO. 61]. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this aspect of the invention.

10 Preferred NAP proteins according to this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6 is an amino acid sequence of 3 to 5 amino acid residues, and A10
15 is an amino acid sequence of 1 to 25 amino acid residues.

Thus, in one preferred aspect, NAPs exhibiting serine protease activity have at least one NAP domain of Formula IV which includes the amino acid sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
20 Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 86 and 254; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 255 to 257; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 258 to 271; (d) Cys-A5 is selected from SEQ. ID. NOS. 272 and 273; (e) Cys-A6 is
25 selected from one of SEQ. ID. NOS. 274 to 276; (f) Cys-A7-Cys-A8 is selected from one of SEQ. ID. NOS. 277 to 279; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 280 to 282; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 283 to 307.

30 In another preferred embodiment, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3 is Glu-Pro-Lys.

In an additional preferred embodiment, A4 is an amino
35 acid sequence having a net anionic charge.

In another preferred embodiment, A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues. Preferably, A5_a is Thr and A5_c is Asn. An especially preferred A5 sequence includes
40 Thr-Leu-Asn or Thr-Met-Asn.

According to this aspect of the invention, a preferred A7 amino acid residue is Gln.

5 In one embodiment of this aspect of the invention, a preferred NAP molecule is one wherein

 (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;

 (b) A4 is an amino acid sequence having a net
10 anionic charge;

 (c) A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues, and

 (d) A7 is Gln. Pharmaceutical compositions
15 comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one
20 NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this embodiment of the invention.

 In another preferred embodiment, a NAP molecule is
25 one wherein

 (a) A3 is Glu-Pro-Lys;

 (b) A4 is an amino acid sequence having a net anionic charge;

 (c) A5 is selected from Thr-Leu-Asn and Thr-Met-Asn;
30 and

 (d) A7 is Gln. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment
35 also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred
40 NAPs according to this embodiment of the invention.

 Preferred NAP proteins having serine protease inhibitory activity, according to all the embodiments

5 recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides*
10 *polygyrus*. Particularly preferred are NAP proteins HpoNAP5 and NamNAP derived from *Heligomosomoides polygyrus* and *Necator americanus*, respectively.

This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein
15 having serine protease inhibitory activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having serine protease inhibitory activity. Preferred cDNAs according to this aspect have nucleotide sequences [SEQ. ID. NO. 14]
20 (HpoNAP5) and [SEQ. ID. NO. 39] (NamNAP) and code for HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

The serine protease inhibitory activity can be determined using any of the assays disclosed in Examples A through F, or any commonly used enzymatic assay for
25 measuring inhibition of serine protease activity. Procedures for a multitude of enzymatic assays can be found in the volumes of Methods of Enzymology or similar reference materials. Preferred NAPs have serine protease inhibitory activity directed toward enzymes in the blood
30 coagulation cascade or toward trypsin/elastase.

NAPs having anticoagulant activity (FORMULA V)

In another aspect of the invention, NAPs of the present invention also include an isolated protein having
35 anticoagulant activity and having one or more NAP domains, wherein each NAP domain includes the sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 ("FORMULA V"), wherein

(a) A1 is an amino acid sequence of 7 to 8 amino
40 acid residues;

(b) A2 is an amino acid sequence;

5 (c) A3 is an amino acid sequence of 3 amino acid residues;
(d) A4 is an amino acid sequence;
(e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
10 (f) A6 is an amino acid sequence;
(g) A7 is an amino acid;
(h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
(i) A9 is an amino acid sequence of 5 to 7 amino acid residues; AND
15 (j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP
20 domain has in total less than about 120 amino acid residues. Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by
25 this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred NAPs include those having at least one NAP domain having an amino acid sequence substantially the same as any of [SEQ.
30 ID. NOS. 40 to 58]. NAP proteins AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO.
35 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this aspect of the invention. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two
40 NAP domains and are preferred NAPs according to this aspect of the invention.

5 Preferred NAP proteins according to this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6 is an amino acid sequence of 3 to 5 amino acid residues, and A10
10 is an amino acid sequence of 5 to 25 amino acid residues.

Preferred NAPs of the present invention according to this aspect include isolated proteins having anticoagulant activity and having at least one NAP domain of formula V which includes the following sequence:

15 Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 87 and 308; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 309 to 311; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 312 to 325; (d) Cys-A5 is
20 selected from SEQ. ID. NOS. 326 and 327; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 328 to 330; (f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 331 to 332; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 333 to 335; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 336 to
25 356.

In another preferred embodiment, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group
30 consisting of Lys, Thr, and Arg. Especially preferred A3 sequences are selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys.

35 In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

According to this aspect of the invention, a preferred A7 amino acid residue is Val or Ile.

Another preferred embodiment of the invention is one
40 in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

(a) A8_a is the first amino acid residue in A8,

5 (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and

(c) A8_c through A8_g are independently selected amino acid residues.

Preferably, A8_c is Gly, A8_d is selected from the
10 group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. A preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp
15 [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

Another preferred embodiment is one in which A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] include the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74] in A10, and
25 are preferred NAPs according to this embodiment of the invention. NAP protein AcaNAP48 [SEQ. ID. NO. 42] includes the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75] in A10 and is a preferred NAP according to this embodiment of the invention. NAP proteins AcaNAP23
30 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], and AceNAP4 [SEQ. ID. NO. 48, 49 AND 62] include the amino acid sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76] and are preferred NAPs according to
35 this embodiment of the invention. NAP proteins AcaNAP45 [SEQ. ID. NOS. 50, 53 AND 63], AcaNAP47 [SEQ. ID. NO. 51, 54 AND 64], AduNAP7 [SEQ. ID. NO. 52, 56 AND 65], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] include the amino acid sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77] and are preferred NAPs
40 according to this embodiment of the invention.

- 5 In one embodiment, a preferred NAP molecule is one wherein
- (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net
- 10 anionic charge;
- (c) A7 is selected from the group consisting of Val and Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO.
- 15 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
- (e) A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
- 20 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP
- 25 proteins according to this embodiment also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40], AcaNAP6 [SEQ.
- 30 ID. NOS. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one
- 35 NAP domain and are preferred NAPs according to this embodiment. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains and are preferred NAPs according to this embodiment.
- 40 In another preferred embodiment, a NAP molecule is one wherein

- 5 (a) A3 is selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;
- 10 (c) A7 is Val or Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78], A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80], A8_a-
15 A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82], wherein at least one of A8_a and A8_b is Glu or Asp;
- (e) A9 is an amino acid sequence of five amino acid residues; and
- 20 (f) A10 includes an amino acid sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].
- Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one
25 NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40], AcaNAP6 [SEQ. ID. NOS. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this embodiment. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains
30 and are preferred NAPs according to this embodiment.

5 Preferred NAP proteins having anticoagulant activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma*
10 *ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*. Particularly preferred are NAP proteins AcaNAP5 [SEQ. ID. NO. 4 and 40], AcaNAP6 [SEQ. ID. NO. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44],
15 AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AcaNAP31 [SEQ. ID. NO. 47] derived from *Ancylostoma caninum*; AceNAP4 [SEQ. ID. NO. 62], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] derived from
20 *Ancylostoma ceylanicum*; and AduNAP7 [SEQ. ID. NO. 65] and AduNAP4 [SEQ. ID. NO. 55] derived from *Ancylostoma duodenale*.

This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein
25 having anticoagulant activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having anticoagulant activity. Preferred cDNAs according to this aspect include AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5], AcaNAP48 [SEQ.
30 ID. NO. 38], AcaNAP23 [SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33], AcaNAP44 [SEQ. ID. NO. 35], AcaNAP31 [SEQ. ID. NO. 34], AduNAP4 [SEQ. ID. NO. 12], AceNAP5 [SEQ. ID. NO. 10], AceNAP7 [SEQ. ID. NO. 11], AceNAP4 [SEQ. ID. NO. 9], AcaNAP45 [SEQ. ID. NO. 36],
35 AcaNAP47 [SEQ. ID. NO. 37], and AduNAP7 [SEQ. ID. NO. 13].

The anticoagulation activity of NAPs within this aspect of the invention can be determined using protocols described herein. Examples B and F present particularly
40 useful methods for assessing a NAP's anticoagulation activity. The procedures described for detecting NAPs having fXa inhibitory activity (Examples A,C) and fVIIa/TF

- 5 inhibitory activity (Example E) also are useful in evaluating a NAP's anticoagulation activity.

Oligonucleotides

- Another aspect of this invention is an
10 oligonucleotide comprising a sequence selected from
 YG109: TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.
88],
 YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO.
89],
15 NAP-1: AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ. ID. NO.
90], and
 NAP-4.RC: TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ. ID.
NO. 91].

- These oligonucleotide sequences hybridize to nucleic acid
20 sequences coding for NAP protein.

- The isolated NAPs of the present invention include those having variations in the disclosed amino acid sequence or sequences, including fragments, naturally occurring mutations, allelic variants, randomly generated
25 artificial mutants and intentional sequence variations, all of which conserve anticoagulant activity. The term "fragments" refers to any part of the sequence which contains fewer amino acids than the complete protein, as for example, partial sequences excluding portions at the
30 amino-terminus, carboxy-terminus or between the amino-terminus and carboxy-terminus of the complete protein.

- The isolated NAPs of the present invention also include proteins having a recombinant amino acid sequence or sequences which conserve the anticoagulant activity of
35 the NAP domain amino acid sequence or sequences. Thus, as used herein, the phrase "NAP protein" or the term "protein" when referring to a protein comprising a NAP domain, means, without discrimination, native NAP protein and NAP protein made by recombinant means. These
40 recombinant proteins include hybrid proteins, such as fusion proteins, proteins resulting from the expression of multiple genes within the expression vector, proteins

5 resulting from expression of multiple genes within the
chromosome of the host cell, and may include a polypeptide
having anticoagulant activity of a disclosed protein
linked by peptide bonds to a second polypeptide. The
recombinant proteins also include variants of the NAP
10 domain amino acid sequence or sequences of the present
invention that differ only by conservative amino acid
substitution. Conservative amino acid substitutions are
defined as "sets" in Table 1 of Taylor, W.R., J. Mol.
Biol., 188:233 (1986). The recombinant proteins also
15 include variants of the disclosed isolated NAP domain
amino acid sequence or sequences of the present invention
in which amino acid substitutions or deletions are made
which conserve the anticoagulant activity of the isolated
NAP domain sequence or sequences.

20 One preferred embodiment of the present invention is
a protein isolated by biochemical methods from the
nematode, *Ancylostoma caninum*, as described in Example 1.
This protein increases the clotting time of human plasma
in the PT and aPTT assays, contains one NAP domain, and is
25 characterized by an N-terminus having the amino acid
sequence, Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-
Asp [SEQ. ID. NO. 92], and a molecular weight of about 8.7
kilodaltons to about 8.8 kilodaltons as determined by mass
spectrometry.

30 Further preferred embodiments of the present
invention include the proteins having anticoagulant
activity made by recombinant methods from the cDNA library
isolated from the nematode, *Ancylostoma caninum*, for
example, AcaNAP5 [SEQ. ID. NO. 4 or 40], AcaNAP6 [SEQ. ID.
35 NO. 6 or 41], Pro-AcaNAP5 [SEQ. ID. NO. 7], Pro-AcaNAP6
[SEQ. ID. NO. 8], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23
[SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25
[SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31
[SEQ. ID. NO. 47], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47
40 [SEQ. ID. NO. 64], and AcaNAPc2 [SEQ. ID. NO. 59];
isolated from the nematode, *Ancylostoma ceylanium*, for
example, AceNAP4 [SEQ. ID. NO. 62], AceNAP5 [SEQ. ID. NO.

5 57], and AceNAP7 [SEQ. ID. NO. 58]; isolated from the
nematode, *Ancylostoma duodenale*, for example, AduNAP4
[SEQ. ID. NO. 55] and AduNAP7 [SEQ. ID. NO. 65]; isolated
from the nematode *Heligmosmoides polygyrus*, for example,
HpoNAP5 [SEQ. ID. NO. 60]; and the nematode *Necator*
10 *americanus*, for example, NamNAP [SEQ. ID. NO. 61]. The
amino acid sequences of these proteins are shown in
Figures 11 and 16 and elsewhere. Each such preferred
embodiment increases the clotting time of human plasma in
the PT and aPTT assays and contains at least one NAP
15 domain.

With respect to "isolated proteins", the proteins of
the present invention are isolated by methods of protein
purification well known in the art, or as disclosed below.
They may be isolated from a natural source, from a
20 chemical mixture after chemical synthesis on a solid phase
or in solution such as solid-phase automated peptide
synthesis, or from a cell culture after production by
recombinant methods.

As described further hereinbelow, the present
25 invention also contemplates pharmaceutical compositions
comprising NAP and methods of using NAP to inhibit the
process of blood coagulation and associated thrombosis.
Oligonucleotide probes useful for identifying NAP nucleic
acid in a sample also are within the purview of the
30 present invention, as described more fully hereinbelow.

1. NAP Isolated From Natural Sources.

The preferred isolated proteins (NAPs) of the present
invention may be isolated and purified from natural
35 sources. Preferred as natural sources are nematodes;
suitable nematodes include intestinal nematodes such as
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus* and *Heligmosomoides*
polygyrus. Especially preferred as a natural source is
40 the hematophagous nematode, the hookworm, *Ancylostoma*
caninum.

5 The preferred proteins of the present invention are isolated and purified from their natural sources by methods known in the biochemical arts. These methods include preparing a soluble extract and enriching the extract using chromatographic methods on different solid
10 support matrices. Preferred methods of purification would include preparation of a soluble extract of a nematode in 0.02 M Tris-HCl, pH 7.4 buffer containing various protease inhibitors, followed by sequential chromatography of the extract through columns containing Concanavalin-A
15 Sepharose matrix, Poros20 HQ cation-ion exchange matrix, Superdex30 gel filtration matrix and a C18 reverse-phase matrix. The fractions collected from such chromatography columns may be selected by their ability to increase the clotting time of human plasma, as measured by the PT and
20 aPTT assays, or their ability to inhibit factor Xa amidolytic activity as measured in a colorimetric amidolytic assay using purified enzyme, or by other methods disclosed in Examples A to F herein. An example of a preferred method of purification of an isolated
25 protein of the present invention would include that as disclosed in Example 1.

 The preferred proteins of the present invention, when purified from a natural source, such as *Ancylostoma caninum*, as described, include those which contain the
30 amino acid sequence: Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-Asp [SEQ. ID. NO. 92]. Especially preferred are the purified proteins having this amino acid sequence at its amino terminus, such as shown in Figure 2 (AcaNAP5 [SEQ. ID. NO. 4]) or Figure 4 (AcaNAP6 [SEQ. ID. NO. 6]).
35 One preferred protein of the present invention was demonstrated to have the amino acid sequence, Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-Asp [SEQ. ID. NO. 92] at its amino-terminus and a molecular weight of 8.7 to 8.8 , kilodaltons, as determined by mass spectrometry.

5 2. NAP Made by Chemical Synthesis.

The preferred isolated NAPs of the present invention may be synthesized by standard methods known in the chemical arts.

10 The isolated proteins of the present invention may be prepared using solid-phase synthesis, such as that described by Merrifield, J. Amer. Chem. Soc., 85:2149 (1964) or other equivalent methods known in the chemical arts, such as the method described by Houghten in Proc. Natl. Acad. Sci., 82:5132 (1985).

15 Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected amino acid or peptide to a suitable insoluble resin. Suitable resins include those containing chloromethyl, bromomethyl, hydroxymethyl, aminomethyl, benzhydryl, and t-
20 alkyloxycarbonylhydrazide groups to which the amino acid can be directly coupled.

In this solid phase synthesis, the carboxy terminal amino acid, having its alpha amino group and, if necessary, its reactive side chain group suitably protected, is first
25 coupled to the insoluble resin. After removal of the alpha amino protecting group, such as by treatment with trifluoroacetic acid in a suitable solvent, the next amino acid or peptide, also having its alpha amino group and, if necessary, any reactive side chain group or groups suitably
30 protected, is coupled to the free alpha amino group of the amino acid coupled to the resin. Additional suitably protected amino acids or peptides are coupled in the same manner to the growing peptide chain until the desired amino acid sequence is achieved. The synthesis may be done
35 manually, by using automated peptide synthesizers, or by a combination of these.

The coupling of the suitably protected amino acid or peptide to the free alpha amino group of the resin-bound amino acid can be carried out according to conventional
40 coupling methods, such as the azide method, mixed anhydride method, DCC (dicyclohexylcarbodiimide) method, activated ester method (p-nitrophenyl ester or N-hydroxysuccinimide

5 'ester), BOP (benzotriazole-1-yl-oxy-tris (diamino)
phosphonium hexafluorophosphate) method or Woodward reagent
K method.

It is common in peptide synthesis that the protecting
groups for the alpha amino group of the amino acids or
10 peptides coupled to the growing peptide chain attached to
the insoluble resin will be removed under conditions which
do not remove the side chain protecting groups. Upon
completion of the synthesis, it is also common that the
peptide is removed from the insoluble resin, and during or
15 after such removal, the side chain protecting groups are
removed.

Suitable protecting groups for the alpha amino group
of all amino acids and the omega amino group of lysine
include benzyloxycarbonyl, isonicotinylloxycarbonyl,
20 o-chlorobenzyloxycarbonyl, p-nitrophenylloxycarbonyl,
p-methoxyphenylloxycarbonyl, t-butoxycarbonyl,
t-amylloxycarbonyl, adamantylloxycarbonyl, 2-(4-biphenyl)-2-
propylloxycarbonyl, 9-fluorenylmethoxycarbonyl,
methylsulfonylethoxycarbonyl, trifluoroacetyl, phthalyl,
25 formyl, 2-nitrophenylsulfonyl, diphenylphosphinothioyl,
dimethylphosphinothioyl, and the like.

Suitable protecting groups for the carboxy group of
aspartic acid and glutamic acid include benzyl ester,
cyclohexyl ester, 4-nitrobenzyl ester, t-butyl ester,
30 4-pyridylmethyl ester, and the like.

Suitable protecting groups for the guanidino group of
arginine include nitro, p-toluenesulfonyl,
benzyloxycarbonyl, adamantylloxycarbonyl,
p-methoxybenzenesulfonyl, 4-methoxy-2,6-
35 dimethylbenzenesulfonyl, 1,3,5-trimethylphenylsulfonyl, and
the like.

Suitable protecting groups for the thiol group of
cysteine include p-methoxybenzyl, triphenylmethyl,
acetylaminomethyl, ethylcarbamoyl, 4-methylbenzyl, 2,4,6-
40 trimethylbenzyl, and the like.

5 Suitable protecting groups for the hydroxy group of serine include benzyl, t-butyl, acetyl, tetrahydropyranyl, and the like.

 The completed peptide may be cleaved from the resin by treatment with liquid hydrofluoric acid containing one or
10 more thio-containing scavengers at reduced temperatures. The cleavage of the peptide from the resin by such treatment will also remove all side chain protecting groups from the peptide.

 The cleaved peptide is dissolved in dilute acetic acid
15 followed by filtration, then is allowed to refold and establish proper disulfide bond formation by dilution to a peptide concentration of about 0.5 mM to about 2 mM in a 0.1 M acetic acid solution. The pH of this solution is adjusted to about 8.0 using ammonium hydroxide and the
20 solution is stirred open to air for about 24 to about 72 hours.

 The refolded peptide is purified by chromatography, preferably by high pressure liquid chromatography on a reverse phase column, eluting with a gradient of
25 acetonitrile in water (also containing 0.1% trifluoroacetic acid), with the preferred gradient running from 0 to about 80% acetonitrile in water. Upon collection of fractions containing the pure peptide, the fractions are pooled and lyophilized to the solid peptide.

30

3. NAP Made By Recombinant Methods.

 Alternatively, the preferred isolated NAPs of the present invention may be made by recombinant DNA methods taught herein and well known in the biological arts.

35 Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual, Second Edition*, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989).

 Recombinant DNA methods allow segments of genetic information, DNA, from different organisms, to be joined
40 together outside of the organisms from which the DNA was obtained and allow this hybrid DNA to be incorporated into

5 a cell that will allow the production of the protein for which the original DNA encodes.

Genetic information encoding a protein of the present invention may be obtained from the genomic DNA or mRNA of an organism by methods well known in the art. Preferred
10 methods of obtaining this genetic information include isolating mRNA from an organism, converting it to its complementary DNA (cDNA), incorporating the cDNA into an appropriate cloning vector, and identifying the clone which contains the recombinant cDNA encoding the desired protein
15 by means of hybridization with appropriate oligonucleotide probes constructed from known sequences of the protein.

The genetic information in the recombinant cDNA encoding a protein of the present invention may be ligated into an expression vector, the vector introduced into host
20 cells, and the genetic information expressed as the protein for which it encodes.

(A) Preparation of cDNA Library.

Preferred natural sources of mRNA from which to
25 construct a cDNA library are nematodes which include intestinal nematodes such as *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* and *Heligmosomoides polygyrus*. Especially preferred as a natural source of mRNA is the hookworm
30 nematode, *Ancylostoma caninum*.

Preferred methods of isolating mRNA encoding a protein of the present invention, along with other mRNA, from an organism include chromatography on poly U or poly T affinity gels. Especially preferred methods of isolating
35 the mRNA from nematodes include the procedure and materials provided in the QuickPrep mRNA Purification kit (Pharmacia).

Preferred methods of obtaining double-stranded cDNA from isolated mRNA include synthesizing a single-stranded
40 cDNA on the mRNA template using a reverse transcriptase, degrading the RNA hybridized to the cDNA strand using a ribonuclease (RNase), and synthesizing a complementary DNA

5 strand by using a DNA polymerase to give a double-stranded
cDNA. Especially preferred methods include those wherein
about 3 micrograms of mRNA isolated from a nematode is
converted into double-stranded cDNA making use of Avian
Myeloblastosis Virus reverse transcriptase, RNase H, and E.
10 coli DNA polymerase I and T4 DNA polymerase.

cDNA encoding a protein of the present invention,
along with the other cDNA in the library constructed as
above, are then ligated into cloning vectors. Cloning
vectors include a DNA sequence which accommodates the cDNA
15 from the cDNA library. The vectors containing the cDNA
library are introduced into host cells that can exist in a
stable manner and provide a environment in which the
cloning vector is replicated. Suitable cloning vectors
include plasmids, bacteriophages, viruses and cosmids.
20 Preferred cloning vectors include the bacteriophages.
Cloning vectors which are especially preferred include the
bacteriophage, lambda gt11 Sfi-Not vector.

The construction of suitable cloning vectors
containing the cDNA library and control sequences employs
25 standard ligation and restriction techniques which are well
known in the art. Isolated plasmids, DNA sequences or
synthesized oligonucleotides are cleaved, tailored and
religated in the form desired.

With respect to restriction techniques, site-specific
30 cleavage of cDNA is performed by treating with suitable
restriction enzyme under conditions which are generally
understood in the art, and the particulars of which are
specified by the manufacturer of these commercially
available restriction enzymes. For example, see the
35 product catalogs of New England Biolabs, Promega and
Stratagene Cloning Systems.

Generally, about 1 microgram of the cDNA is cleaved by
treatment in about one unit of a restriction enzyme in
about 20 microliters of buffer solution. Typically, an
40 excess of restriction enzyme is used to ensure complete
cleavage of the cDNA. Incubation times of about 1 to 2
hours at about 37°C are usually used, though exceptions are

5 known. After each cleavage reaction, the protein may be removed by extraction with phenol/chloroform, optionally followed by chromatography over a gel filtration column, such as Sephadex® G50. Alternatively, cleaved cDNA fragments may be separated by their sizes by
10 electrophoresis in polyacrylamide or agarose gels and isolated using standard techniques. A general description of size separations is found in Methods of Enzymology, 65:499-560 (1980).

The restriction enzyme-cleaved cDNA fragments are then
15 ligated into a cloning vector.

With respect to ligation techniques, blunt-end ligations are usually performed in about 15 to about 30 microliters of a pH 7.5 buffer comprising about 1 mM ATP and about 0.3 to 0.6 (Weiss) units of T4 DNA ligase at
20 about 14°C. Intermolecular "sticky end" ligations are usually performed at about 5 to 100 nanomolar total-end DNA concentrations. Intermolecular blunt-end ligations (usually employing about 10 to 30-fold molar excess of linkers) are performed at about 1 micromolar total-end DNA
25 concentrations.

(B) Preparation of cDNA Encoding NAP.

Cloning vectors containing the cDNA library prepared as disclosed are introduced into host cells, the host cells
30 are cultured, plated, and then probed with a hybridization probe to identify clones which contain the recombinant cDNA encoding a protein of the present invention. Preferred host cells include bacteria when phage cloning vectors are used. Especially preferred host cells include *E. coli*
35 strains such as strain Y1090.

Alternatively, the recombinant cDNA encoding a protein of the present invention may be obtained by expression of such protein on the outer surface of a filamentous phage and then isolating such phage by binding them to a target
40 protein involved in blood coagulation.

An important and well known feature of the genetic code is its redundancy - more than one triplet nucleotide

5 sequence codes for one amino acid. Thus, a number of
different nucleotide sequences are possible for recombinant
cDNA molecules which encode a particular amino acid
sequence for a NAP of the present invention. Such
nucleotide sequences are considered functionally equivalent
10 since they can result in the production of the same amino
acid sequence in all organisms. Occasionally, a methylated
variant of a purine or pyrimidine may be incorporated into
a given nucleotide sequence. However, such methylations do
not affect the coding relationship in any way.

15

(1) Using Oligonucleotide Probes.

Hybridization probes and primers are oligonucleotide
sequences which are complementary to all or part of the
recombinant cDNA molecule that is desired. They may be
20 prepared using any suitable method, for example, the
phosphotriester and phosphodiester methods, described
respectively in Narang, S.A. et al., Methods in Enzymology,
68:90 (1979) and Brown, E.L. et al., Methods in Enzymology,
68:109 (1979), or automated embodiments thereof. In one
25 such embodiment, diethylphosphoramidites are used as
starting materials and may be synthesized as described by
Beaucage et al., Tetrahedron Letters, 22:1859-1862 (1981).
One method for synthesizing oligonucleotides on a modified
solid support is described in U.S. Patent No. 4,458,066.
30 Probes differ from primers in that they are labelled with
an enzyme, such as horseradish peroxidase, or radioactive
atom, such as ^{32}P , to facilitate their detection. A
synthesized probe is radiolabeled by nick translation using
E. coli DNA polymerase I or by end labeling using alkaline
35 phosphatase and T4 bacteriophage polynucleotide kinase.

Preferred hybridization probes include oligonucleotide
sequences which are complementary to a stretch of the
single-stranded cDNA encoding a portion of the amino acid
sequence of a NAP purified from a nematode, such as the
40 hookworm, *Ancylostoma caninum*. For example, a portion of
the amino acid sequence shown in Figure 2 (AcaNAP5) [SEQ.
ID. NO. 4] or Figure 4 (AcaNAP6 [SEQ. ID. NO. 6]) can be

5 used. Especially preferred hybridization probes include those wherein their oligonucleotide sequence is complementary to the stretch of the single-stranded cDNA encoding the amino acid sequence: Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp [SEQ. ID. NO. 93]. Such hybridization
10 probes include the degenerate probe having the oligonucleotide sequence: AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94], wherein R is A or G, Y is T or C, and i is inosine. A preferred recombinant cDNA molecule encoding a protein of the present invention is
15 identified by its ability to hybridize to this probe.

Preferred hybridization probes also include the pair NAP-1 [SEQ. ID. NO. 90] and NAP-4.RC [SEQ. ID. NO. 91], and the pair YG109 [SEQ. ID. NO. 88] and YG103 [SEQ. ID. NO. 89], both of which are described in Examples 13 and 12,
20 respectively.

Upon identification of the clone containing the desired cDNA, amplification is used to produce large quantities of a gene encoding a protein of the present invention in the form of a recombinant cDNA molecule.

25 Preferred methods of amplification include the use of the polymerase chain reaction (PCR). See, e.g., *PCR Technology*, W.H. Freeman and Company, New York (Edit. Erlich, H.A. 1992). PCR is an *in vitro* amplification method for the synthesis of specific DNA sequences. In
30 PCR, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the cDNA of the clone are used. A repetitive series of cycles involving cDNA denaturation into single strands, primer annealing to the single-stranded cDNA, and the extension of the annealed
35 primers by DNA polymerase results in number of copies of cDNA, whose termini are defined by the 5-ends of the primers, approximately doubling at every cycle. *Ibid.*, p.1. Through PCR amplification, the coding domain and any additional primer encoded information such as restriction
40 sites or translational signals (signal sequences, start codons and/or stop codons) of the recombinant cDNA molecule to be isolated is obtained.

5 Preferred conditions for amplification of cDNA include those using Taq polymerase and involving 30 temperature cycles of: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C. Preferred primers include the oligo(dT)-~~Not~~I primer, AATTCGCGGC CGC(T)₁₅ [SEQ. ID. NO. 95], obtained
 10 from Promega Corp. in combination with either (i) the degenerate primer having the oligonucleotide sequence: AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94], wherein R is A or G, Y is T or C, and i is inosine, or (ii) the lambda gt11 primer #1218, GGTGGCGACG ACTCCTGGAG CCCG
 15 [SEQ. ID. NO. 96], obtained from New England Biolabs.

The nucleic acid sequence of a recombinant cDNA molecule made as disclosed is determined by methods based on the dideoxy method of Sanger, F. et al, Proc. Natl. Acad. Sci. USA, 74:5463 (1977) as further described by
 20 Messing, et al., Nucleic Acids Res., 2:309 (1981).

Preferred recombinant cDNA molecules made as disclosed include those having the nucleic acid sequences of Figures 1, 3, 7, 9, 13, and 14.

25 (2) Using NAP cDNAs As Probes.

Also especially preferred as hybridization probes are oligonucleotide sequences encoding substantially all of the amino acid sequence of a NAP purified from the nematode, the hookworm, *Ancylostoma caninum*. Especially
 30 preferred probes include those derived from the AcaNAP5 and AcaNAP6 genes and having the following nucleic acid sequences (AcaNAP5 gene): AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC
 35 TCA CGT GGT TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 1], or Figure 3 (AcaNAP6 gene): AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC
 40 AAG TGC AGT GAG GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA TTC TAC

5 AGA GAC ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC
CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 2].

Preferred hybridization probes also include sequences encoding a substantial part of the amino acid sequence of a NAP, such as the PCR fragment generated with the primer
10 couple NAP-1 [SEQ. ID. NO. 90] and NAP-4.RC [SEQ. ID. NO. 91] as described in Example 13.

(3) Using Phage Display.

Disclosed herein is a method to select cDNAs encoding
15 the proteins of the present invention from whole cDNA libraries making use of filamentous phage display technology. Current display technology with filamentous phage relies on the in-frame insertion of coding regions of interest into gene 3 or gene 8 which code for the
20 attachment protein and major coat protein of the phage, respectively. Those skilled in the art will recognize that various difficulties are inherent in performing this with a vast mixture of cDNAs of unknown sequence and that the most practical way to obtain functional display of
25 cDNA products would consist of fusing the cDNAs through their 5'-end. Indeed, cDNA libraries of sufficient size may contain several cDNAs which derive from the same mRNA but which are 5'-terminally truncated at various positions such that some of them may be expressed as fusion
30 products. A strategy along this line, which relies on the ability of the leucine zippers Jun and Fos to form heterodimers was recently described. See, Cramer, R. and Suter, M., Gene, 137:69-75 (1993).

We have found a novel alternative and direct way to
35 covalently link cDNA gene products to the phage surface; the finding is based on the observation that proteins fused to the C-terminus of phage coat protein 6 can be functionally displayed. This observation has led to the development of a phagemid system as described herein which
40 allows the expression of functionally displayed cDNA products, which in turn permits the affinity-selection of phage particles which contain the cDNA required for the

5 production of the displayed cDNA product. This system provides the basis for the isolation of cDNAs which encode a protein of the present invention. Once isolated, recombinant cDNA molecules containing such cDNA can be used for expression of the proteins of the present
10 invention in other expression systems. The recombinant cDNA molecules made in this way are considered to be within the scope of the present invention.

Recombinant cDNA molecules of the present invention are isolated by preparing a cDNA library from a natural
15 source (as for example, a nematode such as a hookworm), ligating this cDNA library into appropriate phagemid vectors, transforming host cells with these vectors containing the cDNAs, culturing the host cells, infecting the transformed cells with an appropriate helper phage,
20 separating phage from the host cell culture, separating phage expressing a protein of the present invention on its surface, isolating these phage, and isolating a recombinant cDNA molecule from such phage.

The phagemid vectors are constructed using the pUC119
25 expression vector described by Vieira, J. and Messing, J., Methods in Enzymology, 153:3-11 (1987). The filamentous phage gene 6 encoding a surface protein of the phage is modified on its 5' and 3' ends by the addition of HindIII and SfiI restriction sites, respectively, by use of three
30 forward primers and one backward primer using PCR. This results in three DNA fragments which are further modified by addition to their 3' ends of NotI and BamHI restriction sites by PCR. After separate digestion of the three DNA fragments with HindIII and BamHI, the three DNA fragments
35 are ligated into the pUC119 to give pDONG61, pDONG62 and pDONG63 expression vectors. These vectors permit the insertion of cDNA as SfiI-NotI fragments into them.

cDNA libraries are prepared from natural sources, such as nematodes, as described in Examples 2, 9, and 13.
40 Preferred nematodes from which to make such libraries include the intestinal nematodes such as *Ancylostoma*

5 *caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*,
Necator americanus and *Heligmosomoides polygyrus*.

A cDNA library as SfiI-NotI fragments may be directly
directionally ligated into the phagemid vectors pDONG61,
pDONG62 and pDONG63. Alternatively, a cDNA library which
10 has been ligated into the lambda gt11 phage vector as
described in Example 2 can be recovered by PCR, followed
by isolation with electrophoresis and then directional
ligation into these vectors. In the latter approach,
preferred conditions for PCR use Taq polymerase; the
15 primers, lambda gt11 primer #1218 having the sequence
GGTGGCGACG ACTCCTGGAG CCCG (New England Biolabs, Beverly,
MA, USA) [SEQ. ID. NO. 96] and the oligo(dT)-NotI primer
having the sequence, AATTCGCGGC CGC(T)₁₅, (Promega Corp.)
[SEQ. ID. NO. 95]; and 20 temperature cycles of 1 minute
20 at 95°C, 1 minute at 50°C, and 3 minutes at 72°C, followed
by 10 minutes at 65°C.

Host cells are transformed with the pDONG expression
vectors containing a cDNA library. Preferred host cells
include *E. coli* strains, with strain TG1 being especially
25 preferred. Preferred methods for the transformation of *E.*
coli host cells include electroporation.

The transformed cells are cultured at 37°C in LB
medium supplemented with 1% glucose and 100 micrograms/ml
carbenicillin until the optical absorbance at 600 nm
30 reaches the value of 0.5 and then are infected with VCSM13
helper phage (Stratagene) at a multiplicity of infection
(moi) of 20.

The phage are separated from the culture by
centrifugation, then are purified by precipitations with
35 polyethylene glycol/sodium chloride.

The phage which express a NAP of the present
invention on their surface are isolated by taking
advantage of the ability of the NAP to bind to a target
protein involved in blood coagulation, for example, Factor
40 Xa.

Preferred methods of isolating such phage include a
method comprising the steps of:

- 5 (1) combining a solution of factor Xa labelled to biotin with a solution of such phage;
- (2) incubating this mixture;
- (3) contacting a solid phase labelled with streptavidin with this mixture;
- 10 (4) incubating the solid phase with the mixture;
- (5) removing the solid phase from the mixture and contacting the solid phase with buffer to remove unbound phage;
- (6) contacting the solid phase with a second buffer to
- 15 remove the bound phage from the solid phase;
- (7) isolating such phage;
- (8) transforming host cells with such phage;
- (9) culturing the transformed host cells;
- (10) infecting transformed host cells with VCSM13 helper
- 20 phage;
- (11) isolating the phage from the host cell culture; and
- (12) repeating steps (1) to (11) four more times.

An especially preferred method of isolating such phage include the method as detailed in Example 10.

- 25 Single-stranded DNA was prepared from the isolated phages and their inserts 3' to the filamentous phage gene 6 sequenced.

Figure 9 depicts the recombinant cDNA molecule, AcaNAPc2, isolated by the phage display method. The deduced amino acid sequence of the protein of the present invention encoded by AcaNAPc2 is also shown in this figure.

(C) Preparation of Recombinant NAP.

- The recombinant cDNA molecules of the present invention when isolated as disclosed are used to obtain expression of the NAPs of the present invention. Generally, a recombinant cDNA molecule of the present invention is incorporated into an expression vector, this expression vector is introduced into an appropriate host cell, the host cell is cultured, and the expressed protein is isolated.
- 35
 - 40

5 Expression vectors are DNA sequences that are required
for the transcription of cloned copies of genes and
translation of their mRNAs in an appropriate host. These
vectors can express either procaryotic or eucaryotic genes
in a variety of cells such as bacteria, yeast, mammalian,
10 plant and insect cells. Proteins may also be expressed in
a number of virus systems.

Suitably constructed expression vectors contain an
origin of replication for autonomous replication in host
cells, or are capable of integrating into the host cell
15 chromosomes. Such vectors will also contain selective
markers, a limited number of useful restriction enzyme
sites, a high copy number, and strong promoters. Promoters
are DNA sequences that direct RNA polymerase to bind to DNA
and initiate RNA synthesis; strong promoters cause such
20 initiation at high frequency. The preferred expression
vectors of the present invention are operatively linked to
a recombinant cDNA molecule of the present invention, i.e.,
the vectors are capable directing both replication of the
attached recombinant cDNA molecule and expression of the
25 protein encoded by the recombinant cDNA molecule.
Expression vectors may include, but are not limited to
cloning vectors, modified cloning vectors and specifically
designed plasmids or viruses.

Suitable host cells for expression of the proteins of
30 the present invention include bacteria, yeast, mammalian,
plant and insect cells. With each type of cell and species
therein certain expression vectors are appropriate as will
be disclosed below.

Procaryotes may be used for expression of the
35 proteins of the present invention. Suitable bacteria host
cells include the various strains of *E. coli*, *Bacillus*
subtilis, and various species of *Pseudomonas*. In these
systems, plasmid vectors which contain replication sites
and control sequences derived from species compatible with
40 the host are used. Suitable vectors for *E. coli* are
derivatives of pBR322, a plasmid derived from an *E. coli*
species by Bolivar et al., *Gene*, 2:95 (1977). Common

5 procaryotic control sequences, which are defined herein to include promoters for transcription, initiation, optionally with an operator, along with ribosome binding site sequences, include the beta-lactamase and lactose promoter systems (Chang et al., Nature, 198:1056 (1977)), the
10 tryptophan promoter system (Goeddel et al., Nucleic Acids Res., 8:4057 (1980)) and the lambda-derived-P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature, 292:128 (1981)). However, any available promoter system compatible with procaryotes can be used. Preferred
15 procaryote expression systems include *E. coli* and their expression vectors.

Eucaryotes may be used for expression of the proteins of the present invention. Eucaryotes are usually represented by the yeast and mammalian cells. Suitable
20 yeast host cells include *Saccharomyces cerevisiae* and *Pichia pastoris*. Suitable mammalian host cells include COS and CHO (chinese hamster ovary) cells.

Expression vectors for the eucaryotes are comprised of promoters derived from appropriate eucaryotic genes.
25 Suitable promoters for yeast cell expression vectors include promoters for synthesis of glycolytic enzymes, including those for 3-phosphoglycerate kinase gene in *Saccharomyces cerevisiae* (Hitzman et al., J. Biol. Chem., 255:2073 (1980)) and those for the metabolism of methanol
30 as the alcohol oxidase gene in *Pichia pastoris* (Stroman et al., U.S. Patent Nos. 4,808,537 and 4,855,231). Other suitable promoters include those from the enolase gene (Holland, M.J. et al., J. Biol. Chem., 256:1385 (1981)) or the Leu2 gene obtained from YEp13 (Broach, J. et al., Gene,
35 8:121 (1978)).

Preferred yeast expression systems include *Pichia pastoris* and their expression vectors. NAP-encoding cDNAs expressed in *Pichia pastoris* optionally may be mutated to encode a NAP protein that incorporates a proline residue at
40 the C-terminus. In some instances the NAP protein is expressed at a higher level and can be more resistant to

5 unwanted proteolysis. One such cDNA, and its expression in *Pichia pastoris*, is described in Example 17.

Suitable promoters for mammalian cell expression vectors include the early and late promoters from SV40 (Fiers, et al., *Nature*, 273:113 (1978)) or other viral
10 promoters such as those derived from polyoma, adenovirus II, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers may also be incorporated into these expression vectors.

Suitable promoters for plant cell expression vectors
15 include the nopaline synthesis promoter described by Depicker, A. et al., *Mol. Appl. Gen.*, 1:561 (1978).

Suitable promoters for insect cell expression vectors include modified versions of the system described by Smith et al., U.S. Patent No. 4,745,051. The expression vector
20 comprises a baculovirus polyhedrin promoter under whose control a cDNA molecule encoding a protein can be placed.

Host cells are transformed by introduction of expression vectors of the present invention into them. Transformation is done using standard techniques
25 appropriate for each type of cell. The calcium treatment employing calcium chloride described in Cohen, S.N., *Proc. Natl. Acad. Sci. USA*, 69:2110 (1972), or the RbCl method described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, p. 254, Cold Spring Harbor Press (1982)
30 is used for procaryotes or other cells which contain substantial cell wall barriers. The transformation of yeast is carried out as described in Van Solingen, P. et al., *J. Bacter.*, 130:946 (1977) and Hsiao, C.L. et al., *Proc. Natl. Acad. Sci. USA*, 76:3829 (1979). Mammalian
35 cells without much cell wall are transformed using the calcium phosphate procedure of Graham and van der Eb, *Virology*, 52:546 (1978). Plant cells are transformed by infection with *Agrobacterium tumefaciens* as described in Shaw, C. et al, *Gene*, 23:315 (1983). Preferred methods of
40 transforming *E. coli* and *Pichia pastoris* with expression vectors include electroporation.

5 Transformed host cells are cultured under conditions, such as type of media, temperature, oxygen content, fluid motion, etc., well known in the biological arts.

The recombinant proteins of the present invention are isolated from the host cell or media by standard methods
10 well known in the biochemical arts, which include the use of chromatography methods. Preferred methods of purification would include sequential chromatography of an extract through columns containing Poros20 HQ anion-ion exchange matrix or Poros20 HS cation exchange matrix,
15 Superdex30 gel filtration matrix and a C18 reverse-phase matrix. The fractions collected after one such chromatography column may be selected by their ability to increase the clotting time of human plasma, as measured by the PT and aPTT assays, or their ability to inhibit factor
20 Xa amidolytic activity as measured in a colorimetric assay, or demonstration of activity in any of the other assays disclosed herein. Examples of preferred methods of purification of a recombinant protein of the present invention are disclosed in Examples 3, 4, 6, 8, 14 and 15.

25

4. Methods of Using NAP.

In one aspect, the present invention includes methods of collecting mammalian plasma such that clotting of said plasma is inhibited, comprising adding to a blood
30 collection tube an amount of a protein of the present invention sufficient to inhibit the formation of a clot when mammalian blood is drawn into the tube, adding mammalian blood to said tube, separating the red blood cells from the mammalian plasma, and collecting the
35 mammalian plasma.

Blood collection tubes include stoppered test tubes having a vacuum therein as a means to draw blood obtained by venipuncture into the tubes. Preferred test tubes include those which are made of borosilicate glass, and
40 have the dimensions of, for example, 10.25 x 47 mm, 10.25 x 50 mm, 10.25 x 64 mm, 10.25 x 82 mm, 13 x 75 mm, 13 x 100 mm, 16 x 75 mm, 16 x 100 mm or 16 x 125 mm. Preferred

5 stoppers include those which can be easily punctured by a blood collection needle and which when placed onto the test tube provide a seal sufficient to prevent leaking of air into the tube.

10 The proteins of the present invention are added to the blood collection tubes in a variety of forms well known in the art, such as a liquid composition thereof, a solid composition thereof, or a liquid composition which is lyophilized to a solid in the tube. The amount added to such tubes is that amount sufficient to inhibit the
15 formation of a clot when mammalian blood is drawn into the tube. The proteins of the present invention are added to blood collection tubes in such amounts that, when combined with 2 to 10 ml of mammalian blood, the concentration of such proteins will be sufficient to inhibit clot formation.
20 Typically, this effective concentration will be about 1 to 10,000 nM, with 10 to 1000 nM being preferred. Alternatively, the proteins of the present invention may be added to such tubes in combination with other clot-inhibiting additives, such as heparin salts, EDTA salts,
25 citrate salts or oxalate salts.

After mammalian blood is drawn into a blood collection tube containing either a protein of the present invention or the same in combination with other clot-inhibiting additives, the red blood cells are separated from the
30 mammalian plasma by centrifugation. The centrifugation is performed at g-forces, temperatures and times well known in the medical arts. Typical conditions for separating plasma from red blood cells include centrifugation at a centrifugal force of about 100xg to about 1500xg, at a
35 temperatures of about 5 to about 25°C, and for a time of about 10 to about 60 minutes.

The mammalian plasma may be collected by pouring it off into a separate container, by withdrawing it into a pipette or by other means well known to those skilled in
40 the medical arts.

In another aspect, the present invention includes methods for preventing or inhibiting thrombosis (clot

5 formation) or blood coagulation in a mammal, comprising administering to said mammal a therapeutically effective amount of a protein or a pharmaceutical composition of the present invention.

10 The proteins or pharmaceutical compositions of the present invention are administered *in vivo*, ordinarily in a mammal, preferably in a human. In employing them *in vivo*, the proteins or pharmaceutical compositions can be administered to a mammal in a variety of ways, including orally, parenterally, intravenously, subcutaneously, 15 intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Administration is preferably parenteral, such as intravenous on a daily basis. Alternatively, administration is preferably oral, such as by tablets, 20 capsules or elixers taken on a daily basis.

In practicing the methods of the present invention, the proteins or pharmaceutical compositions of the present invention are administered alone or in combination with one another, or in combination with other therapeutic or *in vivo* diagnostic agents. 25

As is apparent to one skilled in the medical art, a therapeutically effective amount of the proteins or pharmaceutical compositions of the present invention will vary depending upon the age, weight and mammalian species 30 treated, the particular proteins employed, the particular mode of administration and the desired affects and the therapeutic indication. Because these factors and their relationship to determining this amount are well known in the medical arts, the determination of therapeutically effective dosage levels, the amount necessary to achieve 35 the desired result of preventing thrombosis, will be within the ambit of one skilled in these arts.

Typically, administration of the proteins or pharmaceutical composition of the present invention is 40 commenced at lower dosage levels, with dosage levels being increased until the desired effect of preventing *in vivo* thrombosis is achieved which would define a therapeutically

5 effective amount. For the proteins of the present
invention, alone or as part of a pharmaceutical
composition, such doses are between about 0.01 mg/kg and
100 mg/kg body weight, preferably between about 0.01 and 10
mg/kg, body weight.

10

5. Utility.

Proteins of the present invention when made and selected
as disclosed are useful as potent inhibitors of blood
coagulation *in vitro* and *in vivo*. As such, these proteins
15 are useful as *in vitro* diagnostic reagents to prevent the
clotting of blood and are also useful as *in vivo*
pharmaceutical agents to prevent or inhibit thrombosis or
blood coagulation in mammals.

The proteins of the present invention are useful as *in*
20 *vitro* diagnostic reagents for inhibiting clotting in blood
drawing tubes. The use of stoppered test tubes having a
vacuum therein as a means to draw blood obtained by
venipuncture into the tube is well known in the medical
arts. Kasten, B.L., "Specimen Collection", Laboratory Test
25 Handbook, 2nd Edition, Lexi-Comp Inc., Cleveland pp. 16-17
(Edits. Jacobs, D.S. et al. 1990). Such vacuum tubes may
be free of clot-inhibiting additives, in which case, they
are useful for the isolation of mammalian serum from the
blood. They may alternatively contain clot-inhibiting
30 additives (such as heparin salts, EDTA salts, citrate salts
or oxalate salts), in which case, they are useful for the
isolation of mammalian plasma from the blood. The proteins
of the present invention are potent inhibitors of blood
clotting and as such, can be incorporated into blood
35 collection tubes to prevent clotting of the mammalian blood
drawn into them.

The proteins of the present invention are used alone,
in combination of other proteins of the present invention,
or in combination with other known inhibitors of clotting,
40 in the blood collection tubes, for example, with heparin
salts, EDTA salts, citrate salts or oxalate salts.

5 The amount to be added to such tubes, or effective amount, is that amount sufficient to inhibit the formation of a blood clot when mammalian blood is drawn into the tube. The proteins of the present invention are added to blood collection tubes in such amounts that, when combined
10 with 2 to 10 ml of mammalian blood, the concentration of such proteins will be sufficient to inhibit the formation of blood clots. Typically, this effective amount is that required to give a final concentration in the blood of about 1 to 10,000 nM, with 10 to 1000 nM being preferred.

15 The proteins of the present invention may also be used to prepare diagnostic compositions. In one embodiment, diagnostic compositions are prepared by dissolving the proteins of the present invention into diagnostically acceptable carriers, which carriers include phosphate
20 buffered saline (0.01 M sodium phosphate + 0.15 M sodium chloride, pH 7.2 or Tris buffered saline (0.05 M Tris-HCl + 0.15 M sodium chloride, pH 8.0). In another embodiment, the proteins of the present invention may be blended with other solid diagnostically acceptable carriers by methods
25 well known in the art to provide solid diagnostic compositions. These carriers include buffer salts.

 The addition of the proteins of the present invention to blood collection tubes may be accomplished by methods well known in the art, which methods include introduction
30 of a liquid diagnostic composition thereof, a solid diagnostic composition thereof, or a liquid diagnostic composition which is lyophilized in such tubes to a solid plug of a solid diagnostic composition.

 The use of blood collection tubes containing the
35 diagnostic compositions of the present invention comprises contacting a effective amount of such diagnostic composition with mammalian blood drawn into the tube. Typically, when a sample of 2 to 10 ml of mammalian blood is drawn into a blood collection tube and contacted with
40 such diagnostic composition therein; the effective amount to be used will include those concentrations of the proteins formulated as a diagnostic composition which in

5 the blood sample are sufficient to inhibit the formation of blood clots. Preferred effective concentrations would be about 1 to 10,000 nM, with 10 to 1000 nM being especially preferred.

10 According to an alternate aspect of our invention, the proteins of the present invention are also useful as pharmaceutical agents for preventing or inhibiting thrombosis or blood coagulation in a mammal. This prevention or inhibition of thrombosis or blood coagulation includes preventing or inhibiting abnormal thrombosis.

15 Conditions characterized by abnormal thrombosis are well known in the medical arts and include those involving the arterial and venous vasculature of mammals. With respect to the coronary arterial vasculature, abnormal thrombosis (thrombus formation) characterizes the rupture
20 of an established atherosclerotic plaque which is the major cause of acute myocardial infarction and unstable angina, and also characterizes the occlusive coronary thrombus formation resulting from either thrombolytic therapy or percutaneous transluminal coronary angioplasty (PTCA).

25 With respect to the venous vasculature, abnormal thrombosis characterizes the condition observed in patients undergoing major surgery in the lower extremities or the abdominal area who often suffer from thrombus formation in the venous vasculature resulting in reduced blood flow to the affected
30 extremity and a predisposition for pulmonary embolism.

Abnormal thrombosis further characterizes disseminated intravascular coagulopathy which commonly occurs within both vascular systems during septic shock, certain viral infections and cancer, a condition wherein there is rapid
35 consumption of coagulation factors and systemic coagulation which results in the formation of life-threatening thrombi occurring throughout the microvasculature leading to widespread organ failure.

The NAP proteins of the present invention also are
40 useful immunogens against which antibodies are raised. Antibodies, both monoclonal and polyclonal, directed to a NAP are useful for diagnostic purposes and for the

5 identification of concentration levels of NAP in various
biological fluids. Immunoassay utilizing these antibodies
may be used as a diagnostic test, such as to detect
infection of a mammalian host by a parasitic worm or to
detect NAP from a parasitic worm in a tissue of the
10 mammalian host. Also, such immunoassays may be used in
the detection and isolation of NAP from tissue
homogenates, cloned cells and the like.

NAP can be used, with suitable adjuvants, as a
vaccine against parasitic worm infections in mammals.
15 Immunization with NAP vaccine may be used in both the
prophylaxis and therapy of parasitic infections. Disease
conditions caused by parasitic worms may be treated by
administering to an animal infected with these parasites
anti-NAP antibody.

20 NAP proteins of this invention having serine protease
inhibitory activity also are useful in conditions or
assays where the inhibition of serine protease is desired.
For example, NAP proteins that inhibit the serine protease
trypsin or elastase are useful for treatment of acute
25 pancreatitis or acute inflammatory response mediated by
leukocytes, respectively.

The recombinant cDNA molecules encoding the proteins
of the present invention are useful in one aspect for
isolating other recombinant cDNA molecules which also
30 encode the proteins of the present invention. In another
aspect, they are useful for expression of the proteins of
the present invention in host cells.

The nucleotide probes of the present invention are
useful to identify and isolate nucleic acid encoding NAPs
35 from nematodes or other organisms. Additionally, the
nucleotide probes are useful diagnostic reagents to detect
the presence of nematode-encoding nucleic acid in a sample,
such as a bodily fluid or tissue from a mammal suspected of
infection by nematode. The probes can be used directly,
40 with appropriate label for detection, to detect the
presence of nematode nucleic acid, or can be used in a more
indirect manner, such as in a PCR-type reaction, to amplify

5 nematode nucleic acid that may be present in the sample for detection. The conditions of such methods and diagnostic assays are readily available in the art.

To assist in understanding, the present invention will now be further illustrated by the following
10 examples. These examples as they relate to this invention should not be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the
15 scope of the invention as described herein and hereinafter claimed.

Examples.

Example 1

20 Isolation of Novel Anticoagulant Protein (NAP) from *Ancylostoma caninum*.

(A) Preparation of the *Ancylostoma caninum* Lysate.

Frozen canine hookworms, *Ancylostoma caninum*, were obtained from Antibody Systems (Bedford, TX). Hookworms
25 were stored at -80°C until used for homogenate.

Hookworms were frozen in liquid nitrogen and ground in a mortar followed by a homogenization on ice in homogenization buffer using a PotterS homogenizer with a teflon piston (B.Braun Melsungen AG, Germany). The
30 homogenization buffer contained: 0.02 M Tris-HCl pH 7.4, 0.05 M NaCl, 0.001 M MgCl₂, 0.001 M CaCl₂, 1.0 x 10⁻⁵ M E-64 protease inhibitor (Boehringer Mannheim, Germany), 1.0 x 10⁻⁵ M pepstatin A (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-6-
35 methylheptanoic acid, ICN Biomedicals, CA), 1.0 x 10⁻⁵ M chymostatin (Boehringer), 1.0 x 10⁻⁵ M leupeptin (ICN), 5 x 10⁻⁵ M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride, ICN), and 5% (v/v) glycerol. Approximately 4 ml of homogenization buffer was used to homogenize each gram of
40 frozen worms (approximately 500 worms). Insoluble material was pelleted by two sequential centrifugation steps: 19,000 x g_{max} at 4°C for 30 minutes followed by

5 110,000 x g_{max} at 4°C for 40 minutes. The supernatant solution was clarified by passage through a 0.45 micrometer cellulose acetate filter (Corning, NY) to give *Ancylostoma caninum* lysate.

10 (B) Concanavalin A Sepharose Chromatography.

Ancylostoma caninum lysate (100 ml) was adsorbed onto 22 ml of Concanavalin A Sepharose (Pharmacia, Sweden) pre-equilibrated with Con A buffer (0.02 M Tris-HCl, pH 7.4, 1 M NaCl, 0.002 M CaCl₂) by loading it onto a 1.6 x
15 11 cm column of this gel at a flow rate of 3 ml/minute (90 cm/hour). The column was at ambient temperature while the reservoir of lysate was maintained at ice bath temperature throughout the procedure. The column was subsequently washed with 2 column volumes of Con A buffer. The column
20 flow-through and wash were collected (approximately 150 ml) and stored at -80°C until further processing was done.

(C) Anion-Exchange Chromatography.

The flow-through and wash of the Concanavalin A
25 Sepharose column was buffered by adding solid sodium acetate to a final concentration of 12.5 mM. The conductivity was reduced by dilution with milliQ water and the pH was adjusted with HCl to pH 5.3. The precipitate formed during pH adjustment was pelleted by centrifugation
30 15,000 x g_{max} at 4°C for 15 minutes. The supernatant solution was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning, NY).

This clarified solution (total volume approximately 600 ml) was loaded on to a Poros20 HQ (Perseptive
35 Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion buffer (0.05 M Na acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the solution added were at ambient temperature throughout this purification step. The column was subsequently washed
40 with 10 column volumes of Anion buffer.

Material that had inhibitory activity, detected following the procedure below, in the factor Xa amidolytic

5 assay was eluted with Cation buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour).

A sample of solution was tested in a factor Xa amidolytic assay as follows. Reaction mixtures (150 microliters) were prepared in 96-well plates containing
10 factor Xa and various dilutions of the sample in assay buffer (100 mM Tris-HCl pH 7.4; 140 mM NaCl; 0.1% BSA). Human factor X was purchased from Enzyme Research Laboratories (South Bend, IN, USA) and activated with
15 Russell's Viper venom using the procedure of Bock, P. E., Craig, P. A., Olson, S. T., and Singh P., Arch. Biochem. Biophys., 273: 375-388 (1989). Following a 30 minute incubation at ambient temperature, the enzymatic reactions were initiated by addition of 50 microliters of a 1 mM substrate solution in water (N-alpha-benzyloxycarbonyl-D-
20 arginyl-L-glycyl-L-arginine p-nitroanilide-dihydrochloride; S-2765; Chromogenix, Mölndal, Sweden) to yield final concentrations of 0.2 nM factor Xa and 0.25 mM S-2765. Substrate hydrolysis was monitored by continuously measuring absorbance at 405 nm using a Vmax
25 kinetic plate reader (Molecular Devices, Menlo Park, CA, USA).

(D) Heat Treatment.

Half of the 0.55 M NaCl elution pool (3 ml) from
30 anion-exchange chromatography was neutralized by adding 1 M Tris-HCl, pH 7.5 to a final concentration of 50 mM, incubated for 5 minutes at 90°C in a glass tube and subsequently cooled rapidly on ice. Insoluble material was pelleted by centrifugation 19,000 x g_{max} at 4°C for 20
35 minutes. The supernatant contained material which inhibited factor Xa in the factor Xa amidolytic assay. About 89% of the factor Xa inhibitory activity was recovered in the supernatant, after this heat treatment after accounting for dilution.

5 (E) Molecular Sieve Chromatography using Superdex30
(alternative for the heat treatment step).

Half of the 0.55 M NaCl elution pool (3 ml) from anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated
10 with 0.01M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. The factor Xa inhibitory activity (determined in the factor Xa amidolytic assay) eluted 56-64 ml into the run (K_{av} of 0.207). This elution volume would be
15 expected for a globular protein with a molecular mass of 14,000 daltons.

(F) Reverse Phase Chromatography.

Hookworm lysate which was fractionated by
20 chromatography on Concanavalin A Sepharose, anion-exchange and Superdex30 (or with the alternative heat treatment step) was loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v)
25 trifluoroacetic acid at a flow rate of 1 ml/minute with a rate of 0.625 % change in acetonitrile/minute. FXa inhibitory activity (determined in the factor Xa amidolytic assay) eluted at approximately 30% acetonitrile. The HPLC runs were performed on a Vista
30 5500 connected with a Polychrom 9600 detector set at 215 nm (Varian, CA). Detector signals were integrated on a 4290 integrator obtained from the same company. Factor Xa inhibitory activity containing fractions were vacuum dried and then redissolved in PBS (0.01 M sodium phosphate, pH
35 7.4, 0.15 M NaCl).

These fractions were pooled and then loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was developed with a linear gradient of 10-35% acetonitrile in 0.1% trifluoroacetic acid at a flow rate
40 of 1 ml/minute with a slower rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity

5 containing fractions were pooled and subsequently vacuum dried.

(G) Molecular Weight Determination of NAP from
Ancylostoma caninum.

10 The estimated mass for NAP isolated as described in this example was determined using electrospray ionisation mass spectrometry.

A vacuum-dried pellet of NAP was dissolved in 50% (v/v) acetonitrile, 1% (v/v) formic acid. Mass analysis
15 was performed using a VG Bio-Q (Fisons Instruments, Manchester UK).

The NAP sample was pumped through a capillary and at its tip a high voltage of 4 kV was applied. Under the influence of the high electric field, the sample was
20 sprayed out in droplets containing the protein molecules. Aided by the drying effect of a neutral gas (N₂) at 60°C, the droplets were further reduced in size until all the solvent had been evaporated and only the protein species remained in the gaseous form. A population of protein
25 species arose which differed from each other in one charge. With a quadrupole analyzer, the different Da/e (mass/charge)-values were detected. Calibration of the instrument was accomplished using Horse Heart Myoglobin (Sigma, Missouri).

30 The estimated mass of NAP isolated as described in sections A, B, C, D, and F of this example is 8734.60 daltons. The estimated mass of native NAP isolated as described in sections A, B, C, E, and F is 8735.67 daltons.

35

(H) Amino Acid Sequencing of NAP from Ancylostoma
caninum.

Amino acid determination was performed on a 476-A Protein/Peptide Sequencer with On Board Microgradient PTH
40 Analyzer and Model 610A Data Analysis System (Applied Biosystems, CA). Quantification of the residues was performed by on-line analysis on the system computer

- 5 (Applied Biosystems, CA); residue assignment was performed by visual analysis of the HPLC chromatograms. The first twenty amino acids of the amino-terminus of native NAP were determined to be:
- 10 Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Asp
Cys Gly Thr Gln Lys Pro [SEQ. ID. NO. 97].

The cysteine residues were not directly detected in this analysis because the sample was not reduced and
15 subsequently alkylated. Cysteines were assigned to the positions where no specific amino acid was identified.

Example 2

Cloning and Sequencing of NAP from *Ancylostoma caninum*.

- 20 (A) Preparation Of Hybridization Probe.

Full-length cDNA clones encoding NAP were isolated by screening a cDNA library, prepared from the mRNA isolated from the nematode, *Ancylostoma caninum*, with a radiolabeled degenerate oligonucleotide whose sequence was
25 based on the first eleven amino acids of the amino-terminus of NAP from *A. caninum*:

Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp [SEQ. ID. NO. 93].

30

The 33-mer oligonucleotide hybridization probe, designated YG99, had the following sequence:

AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO.
35 94]

where "R" refers to A or G; "Y" refers to T or C; and "i" refers to inosine. YG99 was radiolabeled by enzymatic 5'-end phosphorylation (5'-end labeling kit; Amersham,
40 Buckinghamshire, England) using gamma-³²P-ATP (specific activity >7000Ci/mmmole; ICN, Costa Mesa, CA, USA) and

5 subsequently passed over a NAPTM10 column (Pharmacia, Uppsala, Sweden).

(B) Preparation of cDNA Library.

A cDNA library was constructed using described
10 procedures (Promega Protocols and Applications Guide 2nd Ed.; Promega Corp., Madison, WI, USA).

Adult hookworms, *Ancylostoma caninum*, were purchased from Antibody Systems (Bedford, TX). Poly(A+) RNA was prepared using the QuickPrep mRNA Purification Kit
15 (Pharmacia). About 3 micrograms of mRNA were reverse transcribed using an oligo(dT)-NotI primer/adaptor, AATTCGCGCCGC(T)₁₅ [SEQ. ID. NO. 95], (Promega Corp.) and AMV (Avian Myeloblastosis Virus) reverse transcriptase (Boehringer, Mannheim, Germany). The enzymes used for
20 double-stranded cDNA synthesis were the following: *E. coli* DNA polymerase I and RNaseH from Life Technologies (Gaithersburg, MD, USA) and T4 DNA polymerase from Pharmacia.

EcoRI linkers (pCGGAATTCCG) [SEQ. ID. NO. 98] were
25 ligated onto the obtained cDNA after treatment with EcoRI methylase (RiboClone EcoRI Linker Ligation System; Promega).

The cDNAs were digested with NotI and EcoRI, passed over a 1.5% agarose gel (all sizeable material was eluted
30 using the Geneclean protocol, BIO101 Inc., La Jolla, CA), and unidirectionally ligated into the EcoRI-NotI arms of the lambda gt11 Sfi-NotI vector (Promega). After in vitro packaging (GigapackII-Gold, Stratagene, La Jolla, CA) recombinant phage were obtained by infecting strain Y1090
35 (Promega).

The usefulness of the cDNA library was demonstrated by PCR analysis (Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 3 minutes at 72°C) of a number of randomly picked clones
40 using the lambda gt11 primer #1218, having the sequence, GGTGGCGACG ACTCCTGGAG CCCG (New England Biolabs, Beverly, MA, USA) [SEQ. ID. NO. 96]; targeting sequences located

5 upstream of the cDNA insert) in combination with the above-mentioned oligo(dT)-NotI primer/adaptor; the majority of the clones was found to contain cDNA inserts of variable size.

10 (C) Identification of Clones.

Approximately 1×10^6 cDNA clones (duplicate plaque-lift filters were prepared using HybondTM-N; Amersham) were screened with the radiolabeled YG99 oligonucleotide using the following pre-hybridization and hybridization
15 conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times in 2x SSC, 0.1% SDS at 37°C. After exposure (about 72 hours) to
20 X-ray film, a total of between 350 and 500 hybridization spots were identified.

Twenty-four positive clones, designated NAP1 through NAP24, were subjected to a second hybridization round at lower plaque-density; except for NAP24, single plaques
25 containing a homogeneous population of lambda phage were identified. The retained clones were analyzed by PCR amplifications (Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C) using the oligo(dT)-NotI primer (AATTCGCGGC CGC(T)₁₅) [SEQ. ID. NO. 95] in combination
30 with either (i) YG99 or (ii) the lambda gt11 primer #1218. The majority of the clones (20 out of 23) yielded a fragment of about 400 bp when the oligo(dT)-NotI/YG99 primer set was used and a fragment of about 520 bp when
35 the oligo(dT)-NotI/#1218 primer couple was used. Nineteen such possibly full-length clones were further characterized.

The cDNA inserts of five clones were subcloned as SfiI-NotI fragments on both pGEM-5Zf(-) and pGEM-9Zf(-)
40 (Promega). Because the SfiI sites of lambda gt11 and pGEM-5Zf(-) are not compatible with one another, the cloning on this vector required the use of a small adaptor

5 fragment obtained after annealing the following two 5'-end
phosphorylated oligonucleotides: pTGGCCTAGCG TCAGGAGT
[SEQ. ID. NO. 99] and pCCTGACGCTA GGCCATGG [SEQ. ID. NO.
100]. Following preparation of single-stranded DNA, the
10 sequences of these cDNAs were determined with the dideoxy
chain termination method using primer #1233 having the
sequence, AGCGGATAAC AATTTACACAGGA (New England Biolabs)
[SEQ. ID. NO. 101]. All five clones were found to be full-
length including a complete secretion signal. Clones
15 NAP5, NAP7 and NAP22 were found to have an identical
coding region. Clones NAP6 and NAP11 are also identical
but differ from the NAP5 type of coding region. Figure 1
depicts the nucleotide sequence of the NAP5 gene and
Figure 2 depicts the amino acid sequence of the protein
encoded, AcaNAP5. Likewise, Figure 3 depicts the
20 nucleotide sequence of the NAP6 [SEQ. ID. NO. 5] gene and
Figure 4 depicts the amino acid sequence of the protein
encoded, AcaNAP6 [SEQ. ID. NO. 6].

Fourteen other possibly full-length clones were
subjected to a restriction analysis. The above mentioned
25 400 bp PCR product obtained with the YG99/oligo(dT)-NotI
primer couple, was digested with four different enzymes
capable of discriminating between a NAP5- and NAP6-type of
clone: Sau96I, Sau3AI, DdeI, and HpaII. The results were
consistent with 10 out of the 14 clones being NAP5-type
30 (e.g. NAP4, NAP8, NAP9, NAP15, NAP16, NAP17, NAP18, NAP20,
NAP21, and NAP23) while the remaining four were NAP6-type
(e.g. NAP10, NAP12, NAP14, and NAP19).

These clones were renamed to reflect origin from
Ancylostoma caninum by placing the letters Aca immediately
35 before the NAP designation. For example, NAP5 became
AcaNAP5, NAP6 became AcaNAP6 and so forth.

5 Example 3Production and Purification Of Recombinant AcaNAP5 In *P. pastoris*.(A) Expression Vector Construction.

The *Pichia pastoris* yeast expression system,
10 including the *E. coli*/*P. pastoris* shuttle vector, pHILD2,
has been described in a number of United States Patents.
See, e.g., U.S. Patent Nos. 5,330,901; 5,268,273;
5,204,261; 5,166,329; 5,135,868; 5,122,465; 5,032,516;
5,004,688; 5,002,876; 4,895,800; 4,885,242; 4,882,279;
15 4,879,231; 4,857,467; 4,855,231; 4,837,148; 4,818,700;
4,812,405; 4,808,537; 4,777,242; and 4,683,293.

The pYAM7SP8 vector used to direct expression and
secretion of recombinant AcaNAP5 in *P. pastoris* was a
derivative of the pHILD2 plasmid (Despreaux, C.W. and
20 Manning, R.F., Gene 131: 35-41 (1993)), having the same
general structure. In addition to the transcription and
recombination elements of pHILD2 required for expression
and chromosomal integration in *P. pastoris* (see Stroman,
D.W. et al., U.S. Patent No. 4,855,231), this vector
25 contained a chimeric prepro leader sequence inserted
downstream of the alcohol oxidase (AOX1) promoter. The
prepro leader consisted of the *P. pastoris* acid
phosphatase (PHO1) secretion signal fused to a synthetic
19-amino acid pro-sequence. This pro-sequence was one of
30 the two 19-aa pro-sequences designed by Clements et al.,
Gene 106: 267-272 (1991) on the basis of the *Saccharomyces*
cerevisiae alpha-factor leader sequence. Engineered
immediately downstream from the prepro leader sequence was
a synthetic multi-cloning site with recognition sequences
35 for the enzymes StuI, SacII, EcoRI, BglII, NotI, XhoI,
SpeI and BamHI to facilitate the cloning of foreign genes.
NAP as expressed from pYAM7SP8 in *Pichia pastoris* was
first translated as a prepro-product and subsequently
processed by the host cell to remove the pre- and pro-
40 sequences.

The structure of this vector is shown in Figure 12.
The signal sequence (S) has the nucleic acid sequence: ATG

5 TTC TCT CCA ATT TTG TCC TTG GAA ATT ATT TTA GCT TTG GCT
ACT TTG CAA TCT GTC TTC GCT [SEQ. ID. NO. 102]. The pro
sequence (P) has the nucleic acid sequence: CAG CCA GGT
ATC TCC ACT ACC GTT GGT TCC GCT GCC GAG GGT TCT TTG GAC
AAG AGG [SEQ. ID. NO. 103]. The multiple cloning site
10 (MCS) has the nucleic acid sequence: CCT ATC CGC GGA ATT
CAG ATC TGA ATG CGG CCG CTC GAG ACT AGT GGA TCC [SEQ. ID.
NO. 104].

The pGEM-9Zf(-) vector (Promega) containing the
AcaNAP5 cDNA was used to isolate by amplification ("PCR-
15 rescue") the region encoding the mature AcaNAP5 protein
(using Vent polymerase from New England Biolabs, Beverly,
MA; 20 temperature cycles: 1 minute at 94°C, 1 minute at
50°C, and 1.5 minutes at 72°C). The following
oligonucleotide primers were used:

20 YG101: GCTCGCTCTA-GAAGCTTCAG-ACATGTATAA-TCTCATGTTG-G
[SEQ. ID. NO. 105]
YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]

25 The YG101 primer, targeting C-terminal sequences,
contained a non-annealing extension which included XbaI
and HindIII restriction sites (underlined).

Following digestion with XbaI enzyme, the
amplification product, having the expected size, was
30 isolated from gel and subsequently enzymatically
phosphorylated (T4 polynucleotide kinase from New England
Biolabs, Beverly, MA). After heat-inactivation (10
minutes at at 70°C) of the kinase, the blunt-ended/XbaI
fragment was directionally cloned into the vector pYAM7SP8
35 for expression purposes. The recipient vector-fragment
from pYAM7SP8 was prepared by StuI-SpeI restriction, and
purified from agarose gel. The *E. coli* strain, WK6 [Zell,
R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)], was
transformed with the ligation mixture, and ampicillin
40 resistant clones were selected.

Based on restriction analysis, a plasmid clone
containing an insert of the expected size, designated

5 pYAM7SP-NAP5, was retained for further characterization.
Sequence determination of the clone pYAM7SP-NAP5 confirmed
the precise insertion of the mature AcaNAP5 coding region
in fusion with the prepro leader signal, as predicted by
the construction scheme, as well as the absence of
10 unwanted mutations in the coding region.

(B) Expression Of Recombinant AcaNAP5 In *P. pastoris*.

The *Pichia pastoris* strain GTS115 (*his4*) has been
described in Stroman, D.W. et al., U.S. Patent No.
15 4,855,231. All of the *P. pastoris* manipulations were
performed essentially as described in Stroman, D.W. et
al., U.S. Patent No. 4,855,231.

About 1 microgram of pYAM7SP-NAP5 plasmid DNA was
electroporated into the strain GTS115 using a standard
20 electroporation protocol. The plasmid was previously
linearized by SalI digestion, which theoretically
facilitates the targeting and integration of the plasmid
into the *his4* chromosomal locus.

The selection of a AcaNAP5 high-expressor strain was
25 performed essentially as described hereinbelow. His⁺
transformants were recovered on MD plates (Yeast Nitrogen
Base without amino acids (DIFCO), 13.4 g/l; Biotin, 400
micrograms/L; D-glucose, 20 g/l; agar, 15 g/l). Single
colonies (n=60) originating from the electroporation were
30 inoculated into 100 microliters of FM22-glycerol-PTM1
medium in wells of a 96-well plate and were allowed to
grow on a plate-agitator at 30°C for 24 hours. One liter
of FM22-glycerol-PTM1 medium contained 42.87 g KH₂PO₄, 5 g
(NH₄)₂SO₄, 1 g CaSO₄·2H₂O, 14.28 g K₂SO₄, 11.7 g
35 MgSO₄·7H₂O, 50 g glycerol sterilized as a 100 ml solution,
and 1 ml of PTM1 trace mineral mix filter-sterilized. The
FM22 part of the medium was prepared as a 900 ml solution
adjusted to pH 4.9 with KOH and sterile filtered. One
liter of the PTM1 mix contained 6 g CuSO₄·5H₂O, 0.8 g KI,
40 3 g MnSO₄·H₂O, 0.2 g NaMoO₄·2H₂O, 0.02 g H₃BO₃, 0.5 g
CoCl₂·6H₂O, 20 g ZnCl₂, 5 ml H₂SO₄, 65 g FeSO₄·7H₂O, 0.2 g
biotin.

5 The cells were then pelleted and resuspended in fresh
FM22-methanol-PTM1 medium (same composition as above
except that the 50 g glycerol was replaced by 0.5 % (v/v)
methanol in order to induce expression of the AOX1
promoter). After an additional incubation period of 24
10 hours at 30°C, the supernatants of the mini-cultures were
tested for the presence of secreted AcaNAP5. Two clones
that directed a high level of synthesis and secretion of
AcaNAP5, as shown by the appearance of high factor Xa
inhibitory activity in the culture medium (as measured by
15 the amidolytic factor Xa assay described in Example 1),
were selected. After a second screening round, using the
same procedure, but this time at the shake-flask level,
one isolated host cell was chosen and designated *P.*
pastoris GTS115/7SP-NAP5.

20 The host cell, GTS115/7SP-NAP5, was shown to have a
wild type methanol-utilisation phenotype (Mut⁺), which
demonstrated that the integration of the expression
cassette into the chromosome of GTS115 did not alter the
functionality of the genomic AOX1 gene.

25 Subsequent production of recombinant AcaNAP5 material
was performed in shake flask cultures, as described in
Stroman, D.W. et al., U.S. Patent No. 4,855,231. The
recombinant product was purified from *Pichia pastoris* cell
supernatant as described below.

30

(C) Purification of recombinant AcaNAP5.

(1) Cation Exchange Chromatography.

Following expression, the culture supernatant from
GTS115/75SP-NAP5 (100 ml) was centrifuged at 16000 r.p.m.
35 (about 30,000xg) for 20 minutes before the pH was adjusted
with 1N HCl to pH 3. The conductivity of the supernatant
was decreased to less than 10 mS/cm by adding MilliQ
water. The diluted supernatant was clarified by passage
through a 0.22 micrometer cellulose acetate filter
40 (Corning Inc., Corning, NY, USA)

The total volume (approximately 500 ml) of
supernatant was loaded on a Poros20 HS (Perseptive

5 Biosystems, MA) 1 x 2 cm column pre-equilibrated with
Cation Buffer (0.05 M sodium citrate, pH 3) at a flow rate
of 5 ml/minute (400 cm/hour). The column and the sample
were at ambient temperature throughout this purification
step. The column was subsequently washed with 50 column
10 volumes Cation Buffer. Material that had inhibitory
activity in a factor Xa amidolytic assay was eluted with
Cation Buffer containing 1M NaCl at a flow rate of 2
ml/minute.

15 (2) Molecular Sieve Chromatography Using Superdex30.

The 1M NaCl elution pool containing the inhibitory
material (3 ml) from the cation-exchange column was loaded
on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column
pre-equilibrated with 0.01 M sodium phosphate, pH 7.4,
20 0.15 M NaCl at ambient temperature. The chromatography
was conducted at a flow rate of 2 ml/minute. The factor
Xa inhibitory activity eluted 56-64 ml into the run (K_{av}
of 0.207). This is the same elution volume as determined
for the native molecule (Example 1, part E).

25

(3) Reverse Phase Chromatography.

1 ml of the pooled fractions from the gel filtration
chromatography was loaded on to a 0.46 x 25 cm C18 column
(218TP54 Vydac; Hesperia, CA) which was then developed
30 with a linear gradient of 10-35 % acetonitrile in 0.1 %
(v/v) trifluoroacetic acid at 1 ml/minute with a rate of
0.4% change in acetonitrile/minute. Factor Xa inhibitory
activity, assayed as in Example 1, eluted around 30-35%
acetonitrile and was present in several fractions. HPLC
35 runs were performed on the same system as described in
Example 1. Fractions from several runs on this column
containing the factor Xa inhibitory activity were pooled
and vacuum dried.

5 (4) Molecular Weight Determination of Recombinant AcaNAP5

The estimated mass for the main constituent isolated as described in sections (1) to (3) of this example were determined using the same electrospray ionisation mass
10 spectrometry system as described in Example 1.

The estimated mass of recombinant AcaNAP5 was 8735.69 Daltons.

 (5) Amino Acid Sequencing of Recombinant AcaNAP5.

15 Following purification by section (1) to (3) of this example, the recombinant AcaNAP5 from *Pichia pastoris* was subjected to amino acid sequence analysis as described in Example 1. The first five amino acids of the amino-terminus of AcaNAP5 were determined to be: Lys-Ala-Tyr-
20 Pro-Glu [SEQ. ID. NO. 106]. The sequence was identical to the native NAP protein (see Example 1).

Example 4

25 Production and Purification Of Recombinant AcaNAP6 In *P. pastoris*.

(A) Expression Vector Construction.

The expression vector, pYAM7SP-NAP6, was made in the same manner as described for pYAM7SP-NAP5 in Example 3.

30 (B) Expression Of Recombinant AcaNAP6 In *P. pastoris*.

The vector, pYAM7SP-NAP6, was used to transform the *Pichia* strain GTS115 (his4) as described in Example 3.

(C) Purification of AcaNAP6.

35 The recombinant AcaNAP6, expressed from *Pichia* strain GTS115 (his4) transformed with the expression vector, pYAM7SP-NAP6, was purified as described for recombinant AcaNAP5 in Example 3.

The estimated mass of recombinant AcaNAP6 was
40 determined, as described in Example 3, to be 8393.84 Daltons.

5 The majority of the AcaNAP6 preparation had the following amino-terminus: Lys-Ala-Tyr-Pro-Glu [SEQ. ID. NO. 106].

Example 5

10 Expression Of Recombinant Pro-AcaNAP5 In COS Cells

(A) Expression Vector Construction.

 The pGEM-9Zf(-) vector (Promega Corporation, Madison, WI, USA) into which the AcaNAP5 cDNA was subcloned, served as target for PCR-rescue of the entire AcaNAP5 coding
15 region, including the native secretion signal (using Vent polymerase from New England Biolabs, Beverly, MA, USA; 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG101, targeting the 3'-end of the gene
20 encoding a NAP and having the sequence, GCTCGCTCTA GAAGCTTCAG ACATGTATAA TCTCATGTTG G [SEQ. ID. NO. 105], and (2) YG102, targeting the 5'-end of the gene encoding a NAP and having the sequence, GACCAGTCTA GACAATGAAG ATGCTTTACG CTATCG [SEQ. ID. NO. 107]. These primers contain non-
25 annealing extensions which include XbaI restriction sites (underlined).

 Following digestion with XbaI enzyme, the amplification product having the expected size was isolated from an agarose gel and subsequently substituted
30 for the about 450 basepair XbaI stuffer fragment of the pEF-BOS vector [Mizushima, S. and Nagata, S., Nucl. Acids Res., 18: 5322 (1990)] for expression purposes. The recipient vector-fragment was prepared by XbaI digestion and purified from an agarose gel.

35 *E. coli* strain WK6 [Zell, R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)] was transformed with the ligation mixture. Thirty randomly picked ampicillin-resistant transformants were subjected to PCR analysis (Taq polymerase from Life Technologies Inc., Gaithersburg, MD,
40 USA; 30 cycles of amplification with the following temperature program: 1 minute at 95°C, 1 minute at 50°C, and 1 minute at 72°C). Oligonucleotide primers used were:

5 (i) YG103 having the sequence, AAGGCATACC CGGAGTGTGG TG
[SEQ. ID. NO. 89], and matching the amino-terminus of the
region encoding mature NAP, and (ii) YG60 having the
sequence, GTGGGAGACC TGATACTCTC AAG [SEQ. ID. NO. 108],
and targeting vector sequences downstream of the site of
10 insertion, i.e., in the 3'-untranslated region of the pEF-
BOS expression cassette. Only clones that harbor the
insert in the desired orientation can yield a PCR fragment
of predictable length (about 250 basepair). Two such
clones were further characterized by sequence
15 determination and were found to contain the desired XbaI
insert. One of the clones, designated pEF-BOS-NAP5, was
used to transfect COS cells.

(B) Transfection of COS Cells.

- 20 COS-7 cells (ATCC CRL 1651) were transfected with
pEF-BOS-NAP5, pEF-BOS containing an irrelevant insert or
with omission of DNA (mock transfections) using DEAE-
dextran. The following media and stock solutions were
used with the DEAE-dextran method:
- 25 (1) COS-medium: DMEM; 10% FBS (incubated for 30 minutes at
56°C); 0.03% L-glutamine; penicillin (50 I.U./ml) and
streptomycin (50 micrograms/ml) (all products from Life
Technologies).
- (2) MEM-HEPES: MEM medium from Life Technologies Inc.,
30 reconstituted according to the manufacturer's
specifications; containing a 25 mM final concentration of
HEPES; adjusted to pH 7.1 before filtration (0.22
micrometer).
- (3) DNA solution: 6 micrograms DNA per 3 ml MEM-HEPES
- 35 (4) DEAE-dextran solution: 30 microliters DEAE-dextran
stock (Pharmacia, Uppsala, Sweden; 100 mg/ml in H₂O) per 3
ml MEM-HEPES.
- (5) Transfection mixture: 3 ml of the DEAE-dextran
solution is added to 3 ml of the DNA solution and the
40 mixture is left to stand for 30 minutes at ambient
temperature.

- 5 (6) Chloroquine solution: a 1:100 dilution of chloroquine stock (Sigma, St.Louis, MO, USA; 10 mM in water; filtered through a 0.22 micrometer membrane) in COS medium.

Transient transfection of the COS cells was performed as follows. COS cells (about 3.5×10^6), cultured in a
10 175 cm² Nunc TC-flask (Life Technologies Inc.) were washed once with MEM-HEPES. Six ml of the transfection mixture were pipetted onto the washed cells. After incubation for 30 minutes at ambient temperature, 48 ml of the chloroquine solution were added and the cells were
15 incubated for another 4 hours at 37°C. The cells were washed one time with fresh COS-medium and finally incubated in 50 ml of the same medium at 37°C.

(C) Culturing of Transfected COS Cells.

- 20 Three, four, and five days after transfection a sample of the culture supernatants was tested in a factor Xa amidolytic assay according to the procedure in Example 1. The results clearly demonstrated that factor Xa inhibitory activity was accumulating in the culture
25 supernatant of the cells transfected with pEF-BOS-NAP5.

The COS culture supernatant was harvested five days after transfection and the NAP protein purified as described in Example 6.

30 Example 6.

Purification Of Recombinant Pro-AcaNAP5.

(A) Anion Exchange Chromatography.

- The COS culture supernatant containing Pro-AcaNAP5 was centrifuged at 1500 r.p.m. (about 500xg) for 10
35 minutes before adding solid sodium acetate to a final concentration of 50 mM. The following protease inhibitors were added (all protease inhibitors from ICN Biomedicals Inc, Costa Mesa, CA, USA): 1.0×10^{-5} M pepstatin A (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-
40 Ala-4-amino-3-hydroxy-6-methylheptanoic acid), 1.0×10^{-5} M leupeptin, 5×10^{-5} M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride). The pH was adjusted with HCl

5 to pH 5.3. The supernatant was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

The clarified supernatant (total volume approximately 300 ml) was loaded on a Poros20 HQ (Perseptive Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion buffer (0.05 M sodium acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with at least 10 column volumes of Anion buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Anion buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour) and was collected.

20 (B) Molecular Sieve Chromatography Using Superdex30.

The 0.55 M NaCl elution pool (3 ml) from the anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. Material which was inhibitory in the Factor Xa amidolytic assay eluted 56-64 ml into the run (K_{av} of 0.207). This was exactly the same elution volume as determined for the native molecule.

(C) Heat Treatment.

The total pool of fractions having factor Xa inhibitory activity was incubated for 5 minutes at 90°C in a glass tube and subsequently cooled rapidly on ice. Insoluble material was pelleted by centrifugation 19,000 x g_{max} at 4°C for 20 minutes. The supernatant contained all of the factor Xa inhibitory activity.

40 (D) Reverse Phase HPLC Chromatography.

The supernatant of the heat-treated sample was loaded onto a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia,

5 CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at 1 ml/minute with a rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity eluted at approximately 30% acetonitrile. The HPLC runs were
10 performed on the same system as described in Example 1. Factor Xa inhibitory activity-containing fractions were vacuum dried.

(E) Molecular Weight Determination.

15 The estimated mass for recombinant Pro-AcaNAP5, isolated as described in sections A-D of this example, was determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

The estimated mass of recombinant Pro-AcaNAP5
20 was 9248.4 daltons.

(F) Amino Acid Sequencing.

Following purification, the recombinant Pro-AcaNAP5 from COS cells was subjected to amino acid analysis to
25 determine its amino-terminus sequence, as described in Example 1. The first nine amino acids of the amino-terminus of Pro-AcaNAP5 was determined to be: Arg Thr Val Arg Lys Ala Tyr Pro Glu [SEQ. ID. NO. 109]. Compared to the native AcaNAP5 protein (see Example 1), Pro-AcaNAP5
30 possesses four additional amino acids on its N-terminus. The amino acid sequence of Pro-AcaNAP5 is shown in Figure 5.

Example 7

35 Expression Of Recombinant Pro-AcaNAP6 In COS Cells

Pro-AcaNAP6 was transiently produced in COS cells essentially as described for Pro-AcaNAP5 in Example 5.

The AcaNAP6 coding region, including the secretion signal, was PCR-rescued with the same two oligonucleotide
40 primers used for AcaNAP5: (1) YG101 targeting the 3'-end of the gene and having the sequence, GCTCGCTCTA GAAGCTTCAG ACATGTATAA TCTCATGTTG G [SEQ. ID. NO. 105], and (2) YG102

5 targeting the 5'-end of the gene and having the sequence,
GACCAGTCTA GACAATGAAG ATGCTTTACG CTATCG [SEQ. ID. NO.
107]. The YG101-primer contains a non-matching nucleotide
when used with AcaNAP6 as target (underlined T-residue;
compare with Figure 1 and Figure 3); this mismatch results
10 in the replacement an ATT Ile-codon by an ATA Ile-codon.
The mismatch did not markedly influence the amplification
efficiency.

The following modification from Example 5 was
introduced: twenty-four hours after transfection of the
15 COS cells (which is described in Example 5, section B) the
COS-medium containing 10% FBS was replaced with 50 ml of a
medium consisting of a 1:1 mixture of DMEM and Nutrient
Mixture Ham's F-12 (Life Technologies). The cells then
were further incubated at 37°C and the production of
20 factor Xa inhibitory activity detected as described in
Example 5.

Example 8

Purification Of Recombinant Pro-AcaNAP6.

25 (A) Anion Exchange Chromatography.

The COS culture supernatant containing Pro-AcaNAP6
was centrifuged at 1500 r.p.m. for 10 minutes before
adding solid sodium acetate to a final concentration of 50
mM. The following protease inhibitors were added (all
30 protease inhibitors from ICN Biomedicals Inc, Costa Mesa,
CA, USA): 1.0×10^{-5} M pepstatin A (isovaleryl-Val-Val-4-
amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-
6-methylheptanoic acid), 1.0×10^{-5} M leupeptin, 5×10^{-5}
M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride). The
35 pH was adjusted with HCl to pH 5.3. The supernatant was
clarified by passage through a 0.2 micrometer cellulose
acetate filter (Corning Inc., Corning, NY, USA).

The clarified supernatant (total volume approximately
450 ml) was loaded on a Poros20 HQ (Perseptive
40 Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion
buffer (0.05 M Na sodium acetate, pH 5.3, 0.1 M NaCl) at a
flow rate of 10 ml/minute (800 cm/hour). The column and

5 the sample were at ambient temperature throughout this purification step. The column was subsequently washed with at least 10 column volumes of Anion buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Anion buffer containing 0.55 M NaCl
10 at a flow rate of 5 ml/minute (400 cm/hour) and was collected.

(B) Molecular Sieve Chromatography Using Superdex30.

The 0.55 M NaCl elution pool (3 ml) from the anion-
15 exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. Material which was inhibitory in the Factor Xa
20 amidolytic assay eluted 56-64 ml into the run (K_{av} of 0.207). This was exactly the same elution volume as determined for the native NAP.

(C) Reverse Phase HPLC Chromatography.

25 The pooled fractions from the gel filtration were loaded onto a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which then was developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/minute with a
30 rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity (assayed according to Example 1) eluted at approximately 30% acetonitrile. The HPLC runs were performed on the same system as described in Example 1. Factor Xa inhibitory activity containing-fractions
35 were vacuum dried.

(D) Molecular Weight Determination.

The estimated mass for recombinant Pro-AcaNAP6, isolated as described in sections A to C of this example,
40 was determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

5 The estimated mass of recombinant Pro-AcaNAP6 was
8906.9 daltons.

(E) Amino Acid Sequencing.

Following purification, the recombinant Pro-AcaNAP6
10 from COS cells was subjected to amino acid sequence
analysis as described in Example 1. The first five amino
acids of the N-terminus of Pro-AcaNAP6 were determined to
be: Arg Thr Val Arg Lys [SEQ. ID. NO. 110]. Compared to
the native NAP protein (see Example 1), Pro-AcaNAP6
15 possesses four additional amino acids on its amino-
terminus. The amino acid sequence of Pro-AcaNAP6 is shown
in Figure 6 [SEQ. ID. NO. 8].

Example 9

20 The Use of NAP DNA Sequences to Isolate Genes Encoding
Other NAP Proteins.

The AcaNAP5 and AcaNAP6 cDNA sequences (from Example
2) were used to isolate related molecules from other
parasitic species by cross-hybridization.

25 The pGEM-9Zf(-) vectors (Promega) containing the
AcaNAP5 and AcaNAP6 cDNAs were used to PCR-rescue the
regions encoding the mature NAP proteins (Taq polymerase
from Life Technologies; 20 temperature cycles: 1 minute at
95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The
30 oligonucleotide primers used were: (1) YG109, targeting
the C-terminal sequences of cDNA encoding NAP, and having
the sequence, TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.
88], and (2) YG103 having the sequence, AAGGCATACC-
CGGAGTGTGG-TG [SEQ. ID. NO. 89]. The YG109 primer
35 contains a single nucleotide mismatch (underlined T-
residue; compare with the sequences shown in Figures 1 and
3) when used with AcaNAP6 as target. This did not
markedly influence the amplification efficiency. The
correctly sized PCR products (about 230 basepairs) were
40 both isolated from a 1.5% agarose gel. An equimolar
mixture was radiolabeled by random primer extension (T7

5 QuickPrime kit; Pharmacia) and subsequently passed over a Bio-Spin 30 column (Bio-Rad, Richmond, CA, USA).

Ancylostoma ceylanicum (Ace), *Ancylostoma duodenale* (Adu), and *Heligmosomoides polygyrus* (Hpo) cDNA libraries were prepared essentially as described for *Ancylostoma*
10 *caninum* in Example 2.

Ancylostoma ceylanicum and *Heligmosomoides polygyrus* were purchased from Dr. D. I. Pritchard, Department of Life Science, University of Nottingham, Nottingham, UK. *Ancylostoma duodenale* was purchased from Dr. G. A. Schad,
15 The School of Veterinary Medicine, Department of Pathobiology, University of Pennsylvania, Philadelphia, PA, USA.

In each case, the cDNAs were directionally cloned as ECORI-NotI fragments in lambda gt11. Approximately 2×10^5
20 cDNA clones from each library (duplicate plaque-lift filters were prepared using HybondTM-N; Amersham) were screened with the radiolabeled AcaNAP5 and AcaNAP6 fragments using the following prehybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM
25 trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 20% formamide, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times for 30 minutes in 2x SSC, 0.1% SDS at 37°C. After exposure (about 60 hours) to X-ray film, a total of
30 between 100 and 200 hybridization spots were identified in the case of Ace and Adu. A small number of very faint spots were visible in the case of the Hpo cDNA library. For each of the libraries, eight positives were subjected to a second hybridization round at lower plaque-density so
35 as to isolate single plaques.

The retained clones were further characterized by PCR amplification of the cDNA-inserts using the oligo(dT)-NotI primer (Promega; this is the same primer used to prepare first strand cDNA; see Example 2) [SEQ. ID. NO. 95] in
40 combination with the lambda-gt11 primer #1218 having the sequence, GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96] (New England Biolabs; primer #1218 targets lambda

5 sequences located upstream of the site of cDNA insertion).
PCR amplifications were performed as follows: Taq
polymerase from Boehringer; 30 temperature cycles: 1
minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C.
Gel-electrophoretic analysis of the PCR products clearly
10 demonstrated that cDNAs of roughly the same size as the
AcaNAP5 cDNA (e.g., 400 to 500 bp) were obtained for each
species. In addition to these AcaNAP5-sized cDNAs, some
Ace and Adu cDNAs were estimated to be about 700 bp long.

A number of clones, containing either a 500 bp or an
15 800 bp insert, were chosen for sequence determination. To
that end the cDNA inserts were subcloned, as SfiI-NotI
fragments, into pGEM-type phagemids (Promega; refer to
Example 2 for details) which permit the preparation of
single stranded DNA. The sequencing results led to the
20 identification of six different new NAP-like proteins,
designated as follows: AceNAP4, AceNAP5, AceNAP7, AduNAP4,
AduNAP7, and HpoNAP5. The nucleotide sequences of the
cDNAs as well as the deduced amino acid sequences of the
encoded proteins are shown in Figure 7A (AceNAP4 [SEQ. ID.
25 NO. 9]), Figure 7B (AceNAP5) [SEQ. ID. NO. 10], Figure 7C
(AceNAP7) [SEQ. ID. NO. 11], Figure 7D (AduNAP4) [SEQ. ID.
NO. 12], Figure 7E (AduNAP7) [SEQ. ID. NO. 13], and Figure
7F (HpoNAP5) [SEQ. ID. NO. 14]. The AceNAP4 [SEQ. ID. NO.
9] and AduNAP7 [SEQ. ID. NO. 13] cDNAs, each about 700 bp
30 long, each encoded proteins which incorporated two NAP
domains; the other cDNAs isolated coded for a protein
having a single NAP domain. The AduNAP4 cDNA clone [SEQ.
ID. NO. 12] was not full-length, i.e., the clone lacked
the 5'-terminal part of the coding region; the correct
35 reading frame could, however, be assigned based on amino
acid sequence homology with the NAP family of related
molecules.

The identified cDNA sequences can be used to produce
the encoded proteins as disclosed in Examples 3, 4, 5, and
40 7 using the same or alternative suitable expression
systems. Conditioned media or cell lysates, depending on
the system used, can be tested as such or after

- 5 fractionation (using such methodology as outlined in
Example 3, 4, 6 and 8) for protease inhibitory and
anticoagulant activity. Proteins that are encoded by
cDNAs which hybridize to probes derived from fragments of
the AcaNAP5 gene (Figure 1) [SEQ. ID. NO. 3] and/or the
10 AcaNAP6 gene (Figure 3) [SEQ. ID. NO. 5] and that possess
serine protease inhibitory and/or anticoagulant properties
are considered to belong to the NAP family of related
molecules.

5 Example 10Identification of NAP by Functional Display of cDNA
Encoded Proteins.(A) The pDONG Series of Vectors.

- 10 The nucleotide sequences of the pDONG vectors,
pDONG61 [SEQ. ID. NO. 15], pDONG62 [SEQ. ID. NO. 16] and
pDONG63 [SEQ. ID. NO. 17], derivatives of pUC119 [Vieira,
J. and Messing, J., Methods in Enzymology, 153:3-11
(1987)], are depicted in Figures 8A to 8C respectively.
- 15 To construct these three vectors, HindIII and SfiI
restriction sites were added at the 5'-end and 3'-end of
the filamentous phage gene 6 by PCR amplification of the
M13K07 single stranded DNA [Vieira, J. and Messing, J.,
Ibid] with the G6BACKHIND backward primer and G6FORSFI61,
20 G6FORSFI62 or G6FORSFI63 as forward primers. In a second
PCR, the three obtained fragments were re-amplified with
G6BACKHIND and G6FORNOTBAMH as forward primer to append
NotI and BamHI sites at the 3'-end of the fragments. The
sequences of the above mentioned PCR-primers are as
25 follows (restriction sites are underlined):

G6BACKHIND: ATCCGAAGCT TTGCTAACAT ACTGCGTAAT AAG
[SEQ. ID. NO. 111]

30 G6FORSFI61: TATGGGATGG CCGACTTGGC CTCCGCCTGA GCCTCCACCT
TTATCCCAAT CCAAATAAGA [SEQ. ID. NO. 112]

G6FORSFI62: ATGGGATGGC CGACTTGGCC CTCCGCCTGA GCCTCCACCT
TTATCCCAAT CCAAATAAGA [SEQ. ID. NO. 113]

35 G6FORSFI63: TATGGGATGG CCGACTTGGC CGATCCGCCT GAGCCTCCAC
CTTTATCCCA ATCCAAATAA [SEQ. ID. NO. 114]

GAG6FORNOTBAMH: AGGAGGGGAT CCGCGGCCGC GTGATATGGG
40 ATGGCCGACT TGGCC [SEQ. ID. NO. 115]

- Finally, the PCR products were gel-purified, individually
digested with HindIII and BamHI and inserted between the
corresponding sites of pUC119. Sequence determination
45 confirmed that pDONG61, pDONG62, and pDONG63 all contained
the intended insert.

5 The pDONG series of vectors permit the cloning of
cDNAs, as SfiI-NotI fragments. This cloning fuses the
cDNAs in each of the three reading (translation) frames to
the 3'-end of filamentous phage gene 6 which encodes one
of the phage's coat proteins. Infection of a male-
10 specific *E. coli* strain harboring a pDONG-derivative, with
VCSM13 helper phage (Stratagene, La Jolla, CA), results in
the rescuing of pseudo-virions which encapsidate one
specific single strand of the pDONG-derivative and which
may also incorporate a recombinant protein 6 (p6) fusion
15 protein in their coat. cDNAs which are such that the
encoded protein is functionally displayed on the phage
surface as a recombinant p6 fusion protein become
identifiable by means of a panning experiment described
below.

20

(B) Transfer of the *Ancylostoma caninum* cDNA Library from
Lambda gt11 to the pDONG Series of Vectors.

A phage lambda preparation of the pooled *A. caninum*
cDNA clones (about 1×10^6 plaques, see Example 2) was
25 used to PCR-rescue the cDNA inserts (Taq polymerase from
Life Technologies, Gaithersburg, MD, USA; 20 temperature
cycles: 1 minute at 95°C, 1 minute at 50°C, and 3 minutes
at 72°C followed by 10 minutes at 65°C), with the lambda
gt11 primer #1218 having the sequence, GGTGGCGACG
30 ACTCCTGGAG CCCG [SEQ. ID. NO. 96] (New England Biolabs,
Beverly, MA, USA; targeting sequences located upstream of
the cDNA insert) in combination with the oligo(dT)-NotI
primer/adaptor (Promega) used for first strand cDNA
synthesis. Following digestion with the restriction
35 enzymes SfiI and NotI, the whole size-range of
amplification products were recovered from agarose gel.

All fragments were directionally cloned into the
pDONG61, pDONG62, and pDONG63 vectors. The recipient
vector-fragments were prepared by digestion of the CsCl
40 purified vectors with SfiI and NotI and purification with
the "Wizard™ PCR Preps DNA Purification System" (Promega
Corp, Madison, WI, USA).

- 5 *E. coli* strain TG1 [Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual, Second Edition*, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989)] was transformed by electroporation with the pDONG/cDNA ligation mixtures.
- 10 Electrotransformed cells were incubated 1 hour at 37 °C in SOC medium [Sambrook, J. et al., *Ibid.*] and plated on LB-agar containing 0.1% glucose and 100 micrograms/ml carbenicillin (245x245x25 mm plates; Nunc). 2.2×10^6 , 1.6×10^6 , and 1.4×10^6 carbenicillin resistant
- 15 transformants were obtained with pDONG61, pDONG62, and pDONG63, respectively. From each respective library, designated 20L, 21L and 22L, a number of randomly picked transformants were subjected to PCR analysis (Taq polymerase from Life Technologies; 30 cycles of
- 20 amplification with the following temperature program: 1 minute at 95°C, 1 minute at 50°C, and 1 to 3 minutes at 72°C) using two primers that match with sequences flanking the multiple cloning site of pUC119 (primers #1224 having the sequence, CGCCAGGGTT TTCCAGTCA CGAC [SEQ. ID. NO.
- 25 116], and #1233 having the sequence, AGCGGATAAC AATTTACAC AGGA [SEQ. ID. NO. 101]; New England Biolabs). The results showed that the vast majority of the clones contained a cDNA-insert of variable size.

30 (C) Factor Xa Based Affinity-Selection of cDNA Clones Encoding a NAP Protein.

- Phage particles from the 20L, 21L and 22L libraries were rescued as follows: each library was scraped from the plates and grown at 37°C in 100 ml LB medium supplemented
- 35 with 1% glucose and 100 micrograms/ml carbenicillin until the optical absorbance at 600 nm reaches the value of 0.5. After addition of VCSM13 helper phage (Stratagene) at a multiplicity of infection (moi) of 20, the culture was left to stand for 30 minutes at 37°C and then slowly
- 40 shaken for another 30 minutes. The cells were pelleted by centrifugation and resuspended in 250 ml LB medium supplemented with 100 micrograms/ml carbenicillin and 50

5 micrograms/ml kanamycin. These cultures were allowed to grow overnight at 30°C under vigorous agitation. The resulting phage particles were purified by two consecutive precipitations with polyethylene glycol/NaCl and resuspended at 1×10^{13} virions per ml in TRIS-buffered
10 saline (0.05M Tris, 0.15M sodium chloride, pH 7.4) (TBS). Equal amounts of phage particles from the 20L, 21L and 22L were then mixed together.

Human factor Xa (see Example 1 for preparation) was biotinylated with biotin-XX-NHS according to
15 manufacturer's instructions (Pierce). The amidolytic activity of the protease was not affected by this modification as shown by an enzymatic assay using the chromogenic substrate S-2765 (Chromogenix; see Example 1). Streptavidin-coated magnetic beads (Dynal; 1 mg per
20 panning round) were washed three times with TBS and blocked in TBS supplemented with 2% skim milk (Difco) at ambient temperature. After one hour, the magnetic beads were washed twice with TBS before use.

For the first round of panning, 1×10^{13} phage from the
25 pooled libraries were incubated for 75 minutes at 4°C in 200 microliters of TBS buffer supplemented with 250 nM biotinylated factor Xa, 5 mM CaCl_2 and 2% skim milk. After this time, 1 mg blocked streptavidin-coated magnetic beads, resuspended in 200 microliters of TBS containing 5
30 mM CaCl_2 and 2 % skim milk, was added to the phage solution and incubated for 1 hour at 4 °C with gentle agitation. With a magnet (Dynal), the magnetic beads were then rinsed ten times with 500 microliters of TBS containing 0.1% Tween-20. Bound phage were eluted from
35 the magnetic beads by incubating them with 500 microliters of 0.1 M glycine-HCl buffer (pH 2.0) for 10 minutes. The supernatant was neutralized with 150 microliters 1 M Tris-HCl buffer (pH 8.0).

For phage propagation, *E. coli* strain TG1 [Sambrook,
40 J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual, Second Edition*, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989)] was grown at 37°C

5 in 10 ml LB medium until the optical absorbance at 600 nm reached the value of 0.5. The culture was infected with 650 microliters of phage eluted from the magnetic beads and briefly incubated at 37°C with no shaking. After centrifugation, the infected cells were resuspended in 2
10 ml LB medium and plated onto 245x245x25 mm plates filled with LB-agar containing 1% glucose and 100 micrograms/ml carbenicillin. After overnight incubation at 37°C, the cells were scraped from the plates and resuspended in 40 ml LB medium supplemented with 1% glucose and 100
15 micrograms/ml carbenicillin. A cell aliquot corresponding to 15 optical densities at 600 nm was then used to inoculate 100 ml LB medium containing 1% glucose and 100 micrograms/ml carbenicillin. Phage rescue for the next panning round was done as outlined above.

20 For the second panning round, 6×10^{12} phage were incubated during 90 minutes with 1 mg blocked streptavidin-coated magnetic beads in 200 microliters of TBS containing 2.5 mM Ca^{2+} and 2% skim milk (this step was introduced in the procedure to avoid selection of
25 streptavidin-binding clones). After removal of the beads, the same protocol was followed as for round 1. Rounds 3, 4 and 5 were accomplished as round 2, except that the phage input was lowered to 2×10^{12} phage.

Twenty-four individual carbenicillin resistant clones
30 that were isolated after five rounds of panning against biotinylated factor Xa, were then analyzed by ELISA. Streptavidin-coated 96-well plates (Pierce) were blocked for 1 hour with 200 microliters of TBS containing 2% skim milk per well, then were incubated for 1 hour with 100
35 microliters of 20 nM biotinylated factor Xa in TBS per well. For each clone, about 10^{10} phage diluted in 100 microliters TBS containing 2% skim milk and 0.1% Tween-20 were added to the wells. After a 2-hour incubation, the wells were rinsed four times with 200 microliters TBS
40 containing 0.1% Tween-20. Bound phage were visualized by consecutively incubating with a rabbit anti-M13 antiserum (see Example 11), an alkaline phosphatase conjugated anti-

5 rabbit serum (Sigma), and p-nitrophenylphosphate as
substrate (Sigma). Absorbances were taken at 405 nm after
20 minutes. Out of the 24 clones, five bound strongly to
factor Xa. No significant non-specific binding was
observed with these phage when tested in the same ELISA
10 with omission of biotinylated factor Xa.

Single stranded DNA was then prepared from the five
positive clones and the inserts 3' to the gene 6 were
submitted to automated DNA sequencing using the primer
#1224 having the sequence, CGCCAGGGTT TTCCCAGTCA CGAC
15 [SEQ. ID. NO. 116] (New England Biolabs). All five clones
were found to contain the same 470 bp 5'-truncated cDNA
fused in frame to gene 6 in pDONG63. The nucleotide
sequence of this cDNA as well as the deduced amino acid
sequence are depicted in Figure 9 [SEQ. ID. NO. 19]. The
20 cDNA, designated AcaNAPc2, encodes a protein, designated
NAP isoform c2, that belongs to the NAP family of related
proteins.

Example 11

25 Preparation of Antiserum Against M13 Phage.

Antiserum against M13 phage was prepared in rabbits
by subcutaneous injections of about 10^{13} M13K07 phage in
500 microliters of PBS (0.01 M sodium phosphate, pH 7.4 +
0.15 M sodium chloride) combined with an equal volume of
30 adjuvant. The M13K07 phage were CsCl-purified essentially
as described by Glaser-Wuttke, G., Keppner, J., and
Rasched, I., Biochim. Biophys. Acta, 985: 239-247 (1989).
The initial injection was done with Complete Freund's
adjuvant on day 0, followed by subsequent injections with
35 Incomplete Freund's adjuvant on days 7, 14 and 35.
Antiserum was harvested on day 42.

The IgG fraction of the antiserum was enriched by
passage over a Protein A-Sepharose column using conditions
well known in the art.

5 Example 12The Use of AcaNAP5 and AcaNAP6 DNA Sequences to Isolate Additional NAP-Encoding Sequences from *A. caninum*.

The AcaNAP5 and AcaNAP6 cDNA sequences (from Example 2) were used to isolate related molecules from the same
10 parasitic species by cross-hybridization.

The pGEM-9Zf(-) vectors (Promega, Madison, WI) containing the AcaNAP5 and AcaNAP6 cDNAs were used to PCR-rescue the regions encoding the mature NAP proteins (Taq polymerase from Life Technologies (Gaithersburg, MD);
15 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG109, targeting the C-terminal-encoding sequences of cDNA encoding AcaNAP5 and AcaNAP6, and having the sequence, TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.
20 88], and (2) YG103, targeting the N-terminal-encoding sequences of mature AcaNAP5 and AcaNAP6, having the sequence, AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]. The YG109 primer contains a single nucleotide mismatch when used with AcaNAP6 as target (underlined T-residue; compare
25 with the sequence shown in Figure 3 [SEQ. ID. NO. 5]). This mismatch did not markedly influence the amplification efficiency. The correctly sized PCR products (about 230 basepairs) for AcaNAP5 and AcaNAP6 were both isolated from a 1.5% agarose gel. An equimolar mixture was radiolabeled
30 by random primer extension (T7 QuickPrime kit; Pharmacia (Sweden) and subsequently passed over a Bio-Spin 30 column (Bio-Rad, Richmond, CA, USA).

Approximately 750,000 *Ancylostoma caninum* (Aca)cDNA clones (refer to Example 2 (B); duplicate plaque-lift
35 filters were prepared using Hybond™-N; Amersham (Buckinghamshire, England) were screened with the radiolabeled AcaNAP5 and AcaNAP6 cDNA fragments using the following prehybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x
40 Denhardt's solution, 0.5% SDS, 20% formamide, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times for 30

5 minutes in 2x SSC, 0.1% SDS at 37°C. After exposure to X-ray film, a total of about 300 positives were identified.

48 of the 300 positives were subjected to PCR-amplification (Taq polymerase from Boehringer Mannheim, Germany; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C) using the above mentioned YG109 primer, specific for the C-terminus-encoding sequence of AcaNAP5 and AcaNAP6 cDNAs, and primer #1218 which targets lambda-gt11 sequences located upstream of the site of cDNA insertion (New England Biolabs, Beverly, MA; GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96]). 31 out of the 48 positives yielded a PCR product of a size similar to that expected for a AcaNAP5/6-type cDNA.

The remaining 17 positives were used as template for amplification with primer #1218 and an AcaNAPc2 specific primer (e.g., LJ189, targeting the AcaNAPc2 C-terminus and having the sequence GTTTCGAGTT CCGGGATATA TAAAGTCC [SEQ. ID. NO. 117]; refer to Example 10 and Figure 9). None of the clones yielded a PCR product. All 17 positives were then subjected to a second hybridization round at lower plaque-density; single isolated clones were identified in all cases. The 17 isolated cDNA clones were re-analyzed by PCR using the primer couples #1218/YG109 and #1218/LJ189. Three out of the 17 clones yielded an amplification product with the #1218/YG109 primers.

The remaining 14 clones were further analyzed by PCR amplification with the primers #1218 and oligo(dT)-Not (Promega, Madison, WI; this is the same primer used to prepare first strand cDNA; see Example 2). All 14 clones yielded a PCR product. Gel-electrophoretic analysis of the PCR products indicated that some cDNAs were considerably longer than the AcaNAP5 cDNA insert.

Ten clones, including those having the largest cDNA inserts, were chosen for sequence determination. To that end the cDNA inserts were subcloned as SfiI-NotI fragments onto pGEM-type phagemids (Promega, Madison, WI), as described in Example 2. The sequencing identified eight additional NAP protein sequences, designated as follows:

5 AcaNAP23, AcaNAP24, AcaNAP25, AcaNAP31, AcaNAP44,
AcaNAP45, AcaNAP47, and AcaNAP48. Two additional cDNA
clones, designated AcaNAP42 and AcaNAP46, encoded proteins
identical to those encoded by AcaNAP31 [SEQ. ID. NO. 34].
The nucleotide sequences of the cDNAs as well as the
10 deduced amino acid sequences of the encoded proteins are
shown in Figure 13A (AcaNAP23 [SEQ. ID. NO. 31]), Figure
13B (AcaNAP24 [SEQ. ID. NO. 32]), Figure 13C (AcaNAP25
[SEQ. ID. NO. 33]), Figure 13D (AcaNAP31 [SEQ. ID. NO.
34]), Figure 13E (AcaNAP44 [SEQ. ID. NO. 35]), Figure 13F
15 (AcaNAP45 [SEQ. ID. NO. 36]), Figure 13G (AcaNAP47 [SEQ.
ID. NO. 37]), and Figure 13H (AcaNAP48 [SEQ. ID. NO. 38]).
All clones were full-length and included a complete
secretion signal. The AcaNAP45 [SEQ. ID. NO. 36] and
AcaNAP47 [SEQ. ID. NO. 37] cDNAs, each encode proteins
20 which incorporate two NAP domains; the other cDNAs code
for a protein having a single NAP domain.

Example 13

The Use of NAP DNA Sequences to Isolate Sequences Encoding 25 a NAP Protein from *Necator americanus*

The sequences of AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6
[SEQ. ID. NO. 5], AcaNAPc2 [SEQ. ID. NO. 19], AcaNAP23
[SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25
[SEQ. ID. NO. 33], AcaNAP31 [SEQ. ID. NO. 34], AcaNAP44
30 [SEQ. ID. NO. 35], AcaNAP45 [SEQ. ID. NO. 36], AcaNAP47
[SEQ. ID. NO. 37], AcaNAP48 [SEQ. ID. NO. 38], AceNAP4
[SEQ. ID. NO. 9], AceNAP5 [SEQ. ID. NO. 10], AceNAP7 [SEQ.
ID. NO. 11], AduNAP4 [SEQ. ID. NO. 12], AduNAP7 [SEQ. ID.
NO. 13], and HpoNAP5 [SEQ. ID. NO. 14] (see Figures 1, 3,
35 7, and 13) were used to isolate related molecules from the
hematophagous parasite *Necator americanus* by PCR-cloning.

Consensus amino acid sequences were generated from
regions of homology among the NAP proteins. These
consensus sequences were then used to design the following
40 degenerate PCR primers: NAP-1, 5'-AAR-CCN-TGY-GAR-MGG-AAR-
TGY-3' [SEQ. ID. NO. 90] corresponding to the amino acid
sequence NH₂-Lys-Pro-Cys-Glu-(Arg/Pro/Lys)-Lys-Cys [SEQ.

5 ID. NO. 118]; NAP-4.RC, 5'-TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA-
3' [SEQ. ID. NO. 91], corresponding to the sequence NH₂-
Cys-(Val/Ile/Gln)-Cys-(Lys/Asp/Glu/Gln)-(Asp/Glu)-Gly-
(Phe/Tyr)-Tyr [SEQ. ID. NO. 119]. These primers were used
pairwise to generate NAP-specific probes by PCR using N.
10 americanus cDNA as template.

Adult worms, N. americanus, were purchased from Dr.
David Pritchard, University of Nottingham. Poly(A⁺) RNA
was prepared using the QuickPrep mRNA Purification Kit
(Pharmacia, Piscataway, New Jersey). One microgram of mRNA
15 was reverse transcribed using AMV reverse transcriptase
and random hexamer primers (Amersham, Arlington Hills,
IL). One fiftieth of the single-stranded cDNA reaction
product was used as template for ~400 pmole of each of
NAP-1 and NAP-4.RC, with PCR GeneAmp (Perkin Elmer,
20 Norwalk, CT) reagents, on a Perkin-Elmer DNA thermal
cycler. PCR conditions were: cycles 1-3, denaturation at
96 °C for 2 minutes, annealing at 37 °C for 1 minute, and
elongation at 72 °C for 3 minutes (ramp time between 37 °C
and 72 °C was 2 minutes); cycles 4-5, denaturation at 94
25 °C for 1 minute, annealing at 37 °C for 1 minute, and
elongation at 72 °C for 2 minutes (ramp time between 37 °C
and 72 °C was 2 minutes); cycles 6-45, denaturation at 94
°C for 1 minutes, annealing at 37 °C for 1 minute, and
elongation at 72 °C for 2 minutes. Elongation times were
30 incremented by 3 seconds/cycle for cycles 6-45.

PCR amplification of N. americanus cDNA with NAP-1
and NAP-4.RC resulted in an approximately 100 bp
amplification product. The PCR product was labeled with
[α-32P]-dCTP (Amersham) using random primer labeling
35 (Stratagene, La Jolla, CA), and labeled DNA was separated
from unincorporated nucleotides using a Chromaspin-10
column (Clontech, Palo Alto, CA).

A cDNA library was constructed using the following
procedure. Double stranded cDNA was synthesized from 1 µg
40 of N. americanus poly(A⁺) RNA using AMV reverse
transcriptase and random hexamer primers (Amersham,
Arlington Hills, IL). cDNA fragments larger than

5 approximately 300 bp were purified on a 6% polyacrylamide gel and ligated to EcoRI linkers (Stratagene, San Diego, CA) using standard procedures. Linkered cDNA was ligated into EcoRI-cut and dephosphorylated lambda gt10 (Stratagene, San Diego, CA) and packaged using a Gigapack Gold II packaging kit (Stratagene, San Diego, CA).

Prehybridization and hybridization conditions were 6X SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), 0.02 M sodium phosphate pH 6.5, 5X Denhardt's solution, 100 µg/ml sheared, denatured salmon sperm DNA, 0.23% dextran sulfate. Prehybridization and hybridization were at 42 °C, and the filters were washed for 30 minutes at 45 °C with 2X SSC after two prewashes with 2X SSC for 20 minutes. The filters were exposed overnight to X-ray film with two intensifying screens at -70 °C.

20 Approximately 400,000 recombinant phage of the random primed N. americanus library (unamplified) were screened with the NAP-1/NAP-4.RC PCR fragment. About eleven recombinant phage hybridized to this probe, of which four were isolated for nucleotide sequencing analysis. Double stranded sequencing was effected by subcloning the EcoRI cDNA fragments contained in these phage isolates into pBluescript II KS+ vector (Stratagene, San Diego, CA). DNA was sequenced using the Sequenase version 2.0 kit (Amersham, Arlington Hills, IL) and M13 oligonucleotide primers (Stratagene, San Diego, CA).

The four lambda isolates contained DNA that encoded a single 79 amino acid NAP polypeptide that resembles NAP sequences from Ancylostoma spp. and H. polygyrus. The NAP polypeptide from N. americanus has a calculated molecular weight of 8859.6 Daltons. The nucleotide and deduced amino acid sequences are shown in Figure 14.

5 Example 14.Expression Of Recombinant AceNAP4 In COS CellsA. Expression

AceNAP4 was transiently produced in COS cells essentially as described for Pro-AceNAP5 in Example 5 and
10 Pro-AceNAP6 in Example 7.

A pGEM-type phagemid that harbors the AceNAP4 cDNA (from Example 9), served as target for PCR-rescue of the entire AceNAP4 coding region, including the secretion signal, using two XbaI-appending oligonucleotide primers.
15 The primers used were: (1) SHPCR4, targeting the 5'-end of the gene and having the sequence, GACCAGTCTA GACCACCATG GCGGTGCTTT ATTCAGTAGC AATA [SEQ. ID. NO. 120], and (2) SHPCR5, targeting the 3'-end of the gene and having the sequence, GCTCGCTCTA GATTATCGTG AGGTTTCTGG TGCAAAAGTG
20 [SEQ. ID. NO. 121]. The XbaI restriction sites included in the primers are underlined. The primers were used to amplify the AceNAP4 sequence according to the conditions described in Example 5.

Following digestion with XbaI enzyme, the
25 amplification product, having the expected size, was isolated from an agarose gel and subsequently substituted for the about 450 basepair XbaI stuffer fragment of the pEF-BOS vector [Mizushima, S. and Nagata, S., Nucl. Acids Res., 18: 5322 (1990)]. The protocol described in Example
30 5 was followed to yield clone pEF-BOS-AceNAP4, which was first shown to harbor the XbaI-insert in the desired orientation by PCR using primers SHPCR4 and YG60, and subsequently confirmed by sequence determination. This clone was used to transfect COS cells according to the
35 methods in Example 5.

Twenty-four hours after transfection of the COS cells (refer to Example 5, section B) the COS-medium containing 10% FBS was replaced with 50 ml of a medium consisting of a 1:1 mixture of DMEM and Nutrient Mixture Ham's F-12
40 (Life Technologies (Gaithersburg, MD)). The cells were then further incubated at 37°C and the production of EGR-

- 5 factor Xa dependent TF/factor VIIa inhibitory activity
detected as described in Example E.

B. Purification of AceNAP4

1. Anion-exchange chromatography

- 10 The COS culture supernatant from the AceNAP4-
expressing cells was centrifuged at 1500 r.p.m. (about
500xg) for 10 minutes before the following protease
inhibitors (ICN Biomedicals Inc., Costa Mesa, CA) were
added (1.0×10^{-5} M pepstatinA (isovaleryl-Val-Val-4-amino-
15 3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3hydroxy-6-
methylheptanoic acid), 1.0×10^{-5} M AEBSF (4-(2-amonoethyl)-
benzenesulfonyl fluoride). Solid sodium acetate was added
to a final concentration of 50mM before the pH was
adjusted with 1N HCl to pH 5.3. The supernatant was
20 clarified by passage through a 0.22 micrometer cellulose
acetate filter (Corning Inc., Corning, NY, USA).

- The clarified supernatant (total volume aproximately
450ml) was loaded on a Poros20 HQ (Perseptive Biosystems,
MA) 1x2cm column preequilibrated with Anion Buffer (0.05M
25 sodium acetate 0.1M NaCl, pH 5.3) at a flow rate of
5ml/minute. The column and the sample were at ambient
temperature throughout this purification step. The column
was subsequently washed with 10 column volumes of Anion
Buffer and 10 column volumes of 50mM sodium acetate,
30 0.37M NaCl, pH5.3

Material that had EGR-FXa dependent fVIIa/TF
amidolytic inhibitory activity (see Example E) was eluted
with 50mM sodium acetate, 1M NaCl, pH5.3 at a flow of
2ml/minute.

35

2. Reverse-phase chromatography

- An aliquot of the pool of fractions collected after
anion exchange chromatography was loaded onto a 0.46x25cm
C18 column (218TP54 Vydac; Hesperia, CA) which was then
40 developed with a linear gradient of 10-35% acetonitrile in
0.1% (v/v) trifluoroacetic acid at 1ml/minute with a rate
of 0.4% change in acetonitrile/minute. EGR-FXa dependent

- 5 TF/FVIIa amidolytic inhibitory activity (see Example E) was monitored and fractions containing this inhibitory activity were isolated and vacuum-dried.

3. Characterization of recombinant AceNAP4

- 10 The AceNAP4 compound demonstrated SDS-PAGE mobility on a 4-20% gel, consistent with its size predicted from the sequence of the cDNA (Coomassie stained gel of material after RP-chromatography).

15 Example 15

Production and Purification Of Recombinant AcaNAPc2 In P. pastoris.

A. Expression Vector Construction.

- 20 Expression of the AcaNAPc2 gene in P. pastoris was accomplished using the protocol detailed in Example 3 for the expression of AcaNAP5 with the following modifications.

- The pDONG63 vector containing the AcaNAPc2 cDNA,
25 described in Example 10, was used to isolate by amplification ("PCR-rescue") the region encoding mature AcaNAPc2 protein (using Vent polymerase from New England Biolabs, Beverly, MA; 20 temperature cycles: 1 minute at 94°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The
30 following oligonucleotide primers were used:

LJ190: AAAGCAACGA-TGCAGTGTGG-TGAG [SEQ. ID. NO. 122]

- 35 LJ191: GCTCGCTCTA-GAAGCTTCAG-TTTCGAGTTC-CGGGATATAT-AAAGTCC
[SEQ. ID. NO. 123]

The LJ191 primer, targeting C-terminal sequences, contained a non-annealing extension which included XbaI and HindIII restriction sites (underlined).

- 40 Following digestion with XbaI enzyme, the amplification product, having the expected size, was isolated from gel and subsequently enzymatically phosphorylated (T4 polynucleotide kinase from New England

5 Biolabs, Beverly, MA). After heat-inactivation (10 minutes at at 70°C) of the kinase, the blunt-ended/XbaI fragment was directionally cloned into the vector pYAM7SP8 for expression purposes. The recipient vector-fragment from pYAM7SP8 was prepared by StuI-SpeI restriction, and
10 purified from agarose gel. The *E. coli* strain, WK6 [Zell, R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)], was transformed with the ligation mixture, and ampicillin resistant clones were selected.

Based on restriction analysis, a plasmid clone
15 containing an insert of the expected size, designated pYAM7SP-NAPC2, was retained for further characterization. Sequence determination of the clone pYAM7SP-NAPC2 confirmed the precise insertion of the mature AcaNAPc2 coding region in fusion with the prepro leader signal, as
20 predicted by the construction scheme, as well as the absence of unwanted mutations in the coding region.

B. Expression Of Recombinant AcaNAPc2 In *P. pastoris*.

The *Pichia* strain GTS115 (*his4*) has been described in
25 Stroman, D.W. et al., U.S. Patent No. 4,855,231. All of the *P. pastoris* manipulations were performed essentially as described in Stroman, D.W. et al., U.S. Patent No. 4,855,231.

About 1 microgram of pYAM7SP-NAPC2 plasmid DNA was
30 electroporated into the strain GTS115 using a standard electroporation protocol. The plasmid was previously linearized by SalI digestion, theoretically targeting the integration event into the his4 chromosomal locus.

The selection of a AcaNAPc2 high-expresser strain was
35 performed as described in Example 3 for NAP isoform 5 (AcaNAP5) using mini-culture screening. The mini-cultures were tested for the presence of secreted AcaNAPc2 using the fVIIa/TF-EGR-fXa assay (Example E) resulting in the selection of two clones. After a second screening round,
40 using the same procedure, but this time at the shake-flask level, one isolated host cell was chosen and designated *P. pastoris* GTS115/7SP-NAPc2.

5 The host cell, GTS115/7SP-NAPc2, was shown to have a wild type methanol-utilisation phenotype (Mut⁺), which demonstrated that the integration of the expression cassette into the chromosome of GTS115 did not alter the functionality of the genomic AOX1 gene.

10 Subsequent production of recombinant AcaNAPc2 material was performed in shake flask cultures, as described in Stroman, D.W. et al., U.S. Patent No. 4,855,231. The recombinant product was purified from *Pichia pastoris* cell supernatant as described below.

15

C. Purification of recombinant AcaNAPc2

1. Cation Exchange chromatography

20 The culture supernatant (100ml) was centrifuged at 16000 rpm (about 30,000xg) for 20 minutes before the pH was adjusted with 1N HCl to pH 3. The conductivity of the supernatant was decreased to less than 10mS/cm by adding MilliQ water. The diluted supernatant was clarified by passage through a 0.22 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

25 The total volume (approximately 500ml) of the supernatant was loaded onto a Poros20HS (Perseptive Biosystems, MA) 1x2cm column pre-equilibrated with Cation Buffer (50mM sodium citrate pH 3) at a flow-rate of 5ml/minute. The column and the diluted fermentation
30 supernatant were at room temperature throughout this purification step. The column was subsequently washed with 50 column volumes Cation Buffer and 10 column volumes Cation Buffer containing 0.1M NaCl. Material that had inhibitory activity in a prothrombinase assay was eluted
35 with Cation Buffer containing 1M NaCl at a flow rate of 2ml/min.

2. Molecular Sieve Chromatography using Superdex30

40 The 1M NaCl elution pool containing the EGR-fXa-fVIIa/TF inhibitory material (3ml; see Example C) from the cation-exchange column was loaded onto a Superdex30 PG (Pharmacia, Sweden) 1.6x60cm column pre-equilibrated with

5 0.1M sodium phosphate pH7.4, 0.15M NaCl at ambient
temperature. The chromatography was conducted at a flow-
rate of 2 ml/minute. The prothrombinase inhibitory
activity (Example C) eluted 56-64ml into the run and was
pooled.

10

3. Reverse Phase Chromatography

One ml of the pooled fractions from the gel
filtration chromatography was loaded onto a 0.46x25 cm C18
column (218TP54 Vydac; Hesperia, CA) which was then
15 developed with a linear gradient 10-30% acetonitrile in
0.1% (v/v) trifluoroacetic acid with a rate of 0.5% change
in acetonitrile/minute. The major peak which eluted around
20-25% acetonitrile, was manually collected and displayed
prothrombinase inhibitory activity.

20

4. Molecular Mass Determination

The estimated mass for the main constituent isolated
as described in section (1) to (3) of this example was
determined using electrospray ionisation mass
25 spectrometry. The estimated mass of the recombinant
AcaNAPc2 was 9640 daltons, fully in agreement with the
calculated molecular mass of this molecule derived from
the cDNA sequence.

30 Example 16

Expression of AcaNAP42 in *P. pastoris*.

The pGEM-9zf(-) vector (Promega) containing the
AcaNAP42 cDNA (Example 12) was used to isolate the region
encoding the mature AcaNAP42 protein by PCR amplification
35 (using Taq polymerase from Perkin Elmer, Branchburg, New
Jersey; 25 temperature cycles: 1 minute at 94°C, 1
minute at 50°C, and 1 minute at 72°C). The following
oligonucleotide primers were used:

40 oligo3: 5'GAG ACT TTT AAA TCA CTG TGG GAT CAG AAG^{3'}
[SEQ. ID. NO. 124]

5 oligo2: 5'TTC AGG ACT AGT TCA TGG TGC GAA AGT AAT
 AAA^{3'} [SEQ. ID. NO. 125]

The oligo 3 primer, targeting the N-terminal sequence, contained a non-annealing extension which
10 includes DraI restriction site (underlined). The oligo 2 primer, targeting the C-terminal sequence, contained SpeI restriction site.

The NAP amplification product, having the expected approximately 250 bp size, was digested with DraI and SpeI
15 enzymes, purified by extraction with phenol: chloroform: iso-amyl alcohol (25:24:1, volume/volume) and precipitated in ethyl alcohol. The recipient vector-fragment from pYAM7SP8 (Example 3) was prepared by StuI- SpeI restriction, purified by extraction with phenol:
20 chloroform:iso-amyl alcohol (25:24:1, volume/volume) and precipitated in ethyl alcohol. The *E.coli* strain, XL1-Blue [Bullock, W.O., Fernande, J.M., and Short, J.M. Biotechniques 5: 376-379 (1987)], was transformed with the ligation mixture that contained the above DNA fragments,
25 and ampicillin resistant clones were selected.

Based on restriction analysis, a plasmid clone containing an insert of the expected size, designated pYAM7SP8-NAP42, was retained for further characterization. Sequence determination of the clone confirmed correct
30 insertion of the mature coding region in fusion with the PH01/alpha-factor prepro leader signal, as predicted by the construction scheme, as well as the absence of unwanted mutations in the coding region.

About 10 micrograms of pYAM 7SP-NAP 42 plasmid were
35 electroporated into *Pichia* strain GTS115 (*his4*), described in Example 3. The plasmid was previously digested by NotI enzyme, targeting the integration event at the AOX1 chromosomal locus.

The His⁺ transformants were selected as described in
40 Example 3. Single colonies (n=90) from the electroporation were grown in wells of a 96-well plate containing 100 microliters of glycerol-minimal medium for

5 24 hours on a plate-shaker at room temperature. One liter of the glycerol-minimal medium contained 13.4 g Yeast Nitrogen Base without amino acids (DIFCO); 400 micrograms biotin; 10 ml glycerol; and 10 mM potassium phosphate (pH 6.0).

10 The cells were pelleted and resuspended in fresh methanol-minimal medium (same composition as above except that the 10 ml glycerol was replaced by 5 ml methanol) to induce the AOX1 promoter. After an additional incubation period of 24 hours with agitation at room temperature, 10
15 microliters of culture supernatants were tested by the Prothrombin Time Assay (Example B). The presence of secreted AcaNAP42 was detected by the prolongation of the coagulation time of human plasma.

20 Example 17

Expression of AcaNAPc2/Proline in *P. pastoris*.

To enhance stability and the expression level of AcaNAPc2, a mutant cDNA was constructed that encoded an additional proline residue at the C-terminus of the
25 protein (AcaNAPc2/Proline or "AcaNAPc2P"). The expression vector, pYAM7SP8-NAPc2/Proline, was made in the same manner as described in Example 16. The oligo 8 primer is the N-terminal primer with DraI restriction site and the oligo 9 primer is the C-terminal primer containing XbaI
30 site and the amino acid codon, TGG, to add one Proline residue to the C-terminal of the natural form of AcaNAPc2.

oligo 8: 5'GCG TTT AAA GCA ACG ATG CAG TGT GGT G³'
[SEQ. ID. NO. 126]

35 oligo 9: 5'C GCT CTA GAA GCT TCA TGG GTT TCG AGT TCC
GGG ATA TAT AAA GTC³' [SEQ. ID. NO. 127]

Following digestion of the amplification product
40 (approximately 270 bp) with DraI and XbaI, the amplification product was purified and ligated with the vector-fragment from pYAM7SP8 prepared by StuI-SpeI restriction. A plasmid clone containing the

- 5 AcaNAPc2/Proline insert was confirmed by DNA sequencing and designated pYAM7SP8-NAPc2/Proline.

The vector, pYAM7SP8-NAPc2/Proline, was used to transform strain GTS115 (his) as described in Example 16. Transformants were selected and grown according to Example 16. The presence of secreted AcaNAPc2/proline in the growth media was detected by the prolongation of the coagulation time of human plasma (see Example B).

Example 18

15 Alternative Methods of Purifying AcaNAP5, AcaNAPc2 and AcaNAPc2P

(A) AcaNAP5

An alternative method of purifying AcaNAP5 from fermentation media is as follows. Cells were removed from a fermentation of a *Pichia pastoris* strain expressing AcaNAP5, and the media was frozen. The purification protocol was initiated by thawing frozen media overnight at 4°C, then diluting it with approximately four parts Milli Q water to lower the conductivity below 8mS. The pH was adjusted to 3.5, and the media was filtered using a 0.22 µm cellulose acetate filter (Corning Inc., Corning, NY).

The activity of the NAP-containing material was determined in the prothrombin time clotting assay at the beginning of the purification procedure and at each step in the procedure using the protocol in Example B.

The filtered media was applied to a Pharmacia SP-Fast Flow column, at a flow rate of 60 ml/min at ambient temperature, and the column was washed with 10 column volumes of 50 mM citrate/phosphate, pH 3.5. Step elution was performed with 100 mM NaCl, 250 mM NaCl, and then 1000 mM NaCl, all in 50 mM citrate/phosphate, pH 3.5. PT activity was detected in the 250 mM NaCl eluate. The total eluate was dialyzed until the conductivity was below 8mS.

The pH of the material was adjusted to 4.5 with acetic acid, and then applied to a sulfoethyl aspartamide

5 column at ambient temperature. Approximately 10 column
volumes of 50 mM ammonium acetate, pH 4.5/40%
acetonitrile, were used to wash the column. The column
was eluted with 50 mM ammonium acetate, pH 4.5/40%
acetonitrile/ 200 mM NaCl, and the eluate was dialyzed or
10 diafiltered as before.

The eluate was adjusted to 0.1% TFA, applied to a
Vydac C18 protein/peptide reverse phase column at ambient
temperature, and eluted using 0.1% TFA/ 19% acetonitrile,
followed by 0.1% TFA/25% acetonitrile, at a flow rate of 7
15 ml/min. NAP was detected in and recovered from the 0.1%
TFA/25% acetonitrile elution.

(B) AcaNAPc2 and AcaNAPc2P

AcaNAPc2 or AcaNAPc2P can be purified as described
20 above with the following protocol modifications. After
thawing and diluting the media to achieve a conductivity
below 8mS, the pH of the AcaNAPc2-containing media was
adjusted to pH 5.0 using NaOH. The filtered media was
applied to a Pharmacia Q Fast Flow column, at a flow rate
25 of 60 ml/min at ambient temperature, and the column was
washed with 10 column volumes of 50 mM acetic acid, pH
5.0. Step elution was performed with 100 mM NaCl, 250 mM
NaCl, and then 1000 mM NaCl, all in 50 mM acetic acid, pH
5.0. PT activity was detected in the 250 mM NaCl eluate.
30 The total eluate was dialyzed until the conductivity was
below 8mS, and the protocol outlined above was followed
using sulfoethyl aspartamide and RP-HPLC chromatography.

Example A.

35 Factor Xa Amidolytic Assay.

The ability of NAPs of the present invention to act as
inhibitors of factor Xa catalytic activity was assessed by
determining the NAP-induced inhibition of amidolytic
activity catalyzed by the human enzyme, as represented by
40 K_i^* values.

The buffer used for all assays was HBSA (10 mM HEPES,
pH 7.5, 150 mM sodium chloride, 0.1% bovine serum

5 albumin). All reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

The assay was conducted by combining in appropriate wells of a Corning microtiter plate, 50 microliters of HBSA, 50 microliters of the test NAP compound diluted
10 (0.025 - 25nM) in HBSA (or HBSA alone for uninhibited velocity measurement), and 50 microliters of the Factor Xa enzyme diluted in HBSA (prepared from purified human factor X obtained from Enzyme Research Laboratories (South Bend, IN) according to the method described by Bock, P.E.
15 et al., Archives of Biochem. Biophys. 273: 375 (1989). The enzyme was diluted into HBSA prior to the assay in which the final concentration was 0.5 nM). Following a 30 minute incubation at ambient temperature, 50 microliters of the substrate S2765 (N-alpha-benzyloxycarbonyl-D-
20 argininy-L-glycyl-L-arginine-p-nitroanilide dihydrochloride, obtained from Kabi Diagnostica (or Kabi Pharmacia Hepar Inc., Franklin, OH) and made up in deionized water followed by dilution in HBSA prior to the assay) were added to the wells yielding a final total
25 volume of 200 microliters and a final concentration of 250 micromolar (about 5-times K_m). The initial velocity of chromogenic substrate hydrolysis was measured by the change in absorbance at 405nm using a Thermo Max[®] Kinetic Microplate Reader (Molecular Devices, Palo alto, CA) over
30 a 5 minute period in which less than 5% of the added substrate was utilized.

Ratios of inhibited pre-equilibrium, steady-state velocities containing NAP (V_i) to the uninhibited velocity of free fXa alone (V_o) were plotted against the
35 corresponding concentrations of NAP. These data were then directly fit to an equation for tight-binding inhibitors [Morrison, J.F., and Walsh, C.T., Adv. Enzymol. 61:201-300 (1988)], from which the apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

40 Table 1 below gives the K_i^* values for the test compounds AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6], and AcaNAPc2 [SEQ. ID. NO. 59], prepared as described

5 in Examples 3, 4, and 15, respectively. The data show the utility of AcaNAP5 and AcaNAP6 as potent in vitro inhibitors of human FXa. In contrast, AcaNAPc2 did not effectively inhibit FXa amidolytic activity indicating that it does not affect the catalytic activity of free
10 fXa.

Table 1

Compound	Ki* (pM)
AcaNAP5	43 ± 5
AcaNAP6	996 ± 65
AcaNAPc2	NI ^a

^aNI=no inhibition; a maximum of 15%
15 inhibition was observed up to 1μM.

Example B.

Prothrombin Time (PT) and Activated Partial Thromboplastin
20 Time (aPTT) Assays.

The ex vivo anticoagulant effects of NAPs of the present invention in human plasma were evaluated by measuring the prolongation of the activated partial thromboplastin time (aPTT) and prothrombin time (PT) over
25 a broad concentration range of each inhibitor.

Fresh frozen pooled normal citrated human plasma was obtained from George King Biomedical, Overland Park, KS. Respective measurements of aPTT and PT were made using the Coag-A-Mate RA4 automated coagulometer (General
30 Diagnostics, Organon Technica, Oklahoma City, OK) using the Automated aPTT Platelin® L reagent (Organon Technica, Durham, NC) and Simplastin® Excel (Organon Technica, Durham, NC) respectively, as initiators of clotting according to the manufacturer's instructions.

35 The assays were conducted by making a series of dilutions of each tested NAP in rapidly thawed plasma followed by adding 200 microliters or 100 microliters of

5 the above referenced reagents to the wells of the assay carousel for the aPTT or PT measurements, respectively. Alternatively, the NAPs were serially diluted into HBSA and 10 µl of each dilution were added to 100µl of normal human plasma in the wells of the Coag-A-Mate assay
10 carousel, followed by addition of reagent.

Concentrations of NAP were plotted against clotting time, and a doubling time concentration was calculated, i.e., a specified concentration of NAP that doubled the control clotting time of either the PT or the aPTT. The
15 control clotting times (absence of NAP) in the PT and APTT were 12.1 seconds and 28.5 seconds, respectively.

Table 2 below shows the ex vivo anticoagulant effects of AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6], AcaNAPc2 [SEQ. ID. NO. 59], and AceNAP4 [SEQ. ID. NO. 62]
20 and Pro-AcaNAP5 [SEQ. ID. NO. 7] represented by the concentration of each that doubled (doubling concentration) the control clotting time of normal human plasma in the respective PT and APTT clotting assays relative to a control assay where no such NAP was present.
25 The data show the utility of these compounds as potent anticoagulants of clotting human plasma. The data also demonstrate the equivalency of native NAP and recombinant NAP.

5

Table 2

Compound	Doubling Concentra- tion (nM) in the PT	Doubling Concentration (nM) in the aPTT
AcaNAP5 ^a	43 ± 8	87 ± 4
AcaNAP6 ^a	37 ± 3	62 ± 0
AcaNAPc2 ^a	15 ± 1	105 ± 11
AceNAP4 ^a	40 ± 4	115 ± 12
AcaNAP5 ^b	26.9	76.2
AcaNAP5 ^c	39.2	60.0
Pro-AcaNAP5 ^d	21.9	31.0

^aMade in *Pichia pastoris*.^bNative protein.^cMade in *Pichia pastoris* (different recombinant batch than (a)).^dMade in COS cells.

10

Figures 10A and 10B also show NAP-induced
prolongation of the PT (Figure 10A) and aPTT (Figure 10B)
15 in a dose-dependent manner.

Example CProthrombinase inhibition assay

The ability of NAP of the present invention to act as
20 an inhibitor of the activation of prothrombin by Factor Xa
that has been assembled into a physiologic prothrombinase
complex was assessed by determining the respective
inhibition constant, K_i^* .

Prothrombinase activity was measured using a coupled
25 amidolytic assay, where a preformed complex of human FXa,
human Factor Va (FVa), and phospholipid vesicles first
activates human prothrombin to thrombin. The amidolytic
activity of the generated thrombin is measured
simultaneously using a chromogenic substrate. Purified
30 human FVa was obtained from Haematologic Technologies,

5 Inc. (Essex Junction, VT). Purified human prothrombin was purchased from Celsus Laboratories, Inc. (Cincinnati, OH). The chromogenic substrate Pefachrome t-PA ($\text{CH}_3\text{SO}_2\text{-D-hexahydrotyrosine-glycyl-L-arginine-p-nitroanilide}$) from Pentapharm Ltd (Basel, Switzerland) was purchased from
10 Centerchem, Inc. (Tarrytown, NY). The substrate was reconstituted in deionized water prior to use. Phospholipid vesicles were made, consisting of phosphotidyl choline (67%, w/v), phosphatidyl glycerol (16%, w/v), phosphatidyl ethanolamine (10%, w/v), and
15 phosphatidyl serine (7%, w/v) in the presence of detergent, as described by Ruf et al. [Ruf, W., Miles, D.J., Rehemtulla, A., and Edgington, T.S. Methods in Enzymology 222: 209-224 (1993)]. The phospholipids were purchased from Avanti Polar Lipids, (Alabaster, Alabama).

20 The prothrombinase complex was formed in a polypropylene test tube by combining FVa, FXa, and phospholipid vesicles (PLV) in HBSA containing 3 mM CaCl_2 for 10 min. In appropriate wells of a microtiter plate, 50 μl of the complex were combined with 50 μl of NAP
25 diluted in HBSA, or HBSA alone (for V_0 (uninhibited velocity) measurement). Following an incubation of 30 min at room temperature, the triplicate reactions were initiated by the addition of a substrate solution, containing human prothrombin and the chromogenic substrate
30 for thrombin, Pefachrome tPA. The final concentration of reactants in a total volume of 150 μL of HBSA was: NAP (.025-25 nM), FXa (250 fM), PLV (5 μM), prothrombin (250 nM), Pefachrome tPA (250 μM , 5X K_m), and CaCl_2 (3 mM).

The prothrombinase activity of fXa was measured as
35 an increase in the absorbance at 405 nm over 10 min (velocity); exactly as described in Example A, under steady-state conditions. The absorbance increase was sigmoidal over time, reflecting the coupled reactions of the activation of prothrombin by the FXa-containing
40 prothrombinase complex, and the subsequent hydrolysis of Pefachrome tPA by the generated thrombin. The data from each well of a triplicate were combined and fit by

5 reiterative, linear least squares regression analysis, as
a function of absorbance versus time², as described
[Carson, S.D. Comput. Prog. Biomed. 19: 151-157 (1985)] to
determine the initial velocity (V_i) of prothrombin
activation. Ratios of inhibited steady-state initial
10 velocities containing NAP (V_i) to the uninhibited velocity
of prothrombinase fXa alone (V_0) were plotted against the
corresponding concentrations of NAP. These data were
directly fit to the equation for tight-binding
inhibitors, as in Example A above, and the apparent
15 equilibrium dissociation inhibitory constant K_i^* was
calculated.

Table 3 below gives the dissociation inhibitor
constant (K_i^*) of recombinant AcaNAP5 [SEQ. ID. NO. 4],
AcaNAP6 [SEQ. ID. NO. 6] and AcaNAPc2 [SEQ. ID. NO. 59]
20 (all made in *Pichia pastoris* as described) against the
activation of prothrombin by human fXa incorporated into a
prothrombinase complex. These data show the utility of
these compounds as inhibitors of human FXa incorporated
into the prothrombinase complex.

25

Table 3

Compound	K_i^* (pM)
AcaNAP5	144 ± 15
AcaNAP6	207 ± 40
AcaNAPc2	2385 ± 283

The data presented in Examples A, B, and C suggest
30 that AcaNAP5 and AcaNAP6 may be interacting with FXa in a
similar manner that involves directly restricting access
of both the peptidyl and macromolecular substrate
(prothrombin) to the catalytic center of the enzyme. In
contrast, AcaNAPc2 appears to be interacting with FXa in a
35 way that only perturbs the macromolecular interactions of
this enzyme with either the substrate and/or cofactor

- 5 (Factor Va), while not directly inhibiting the catalytic turnover of the peptidyl substrate (see Table 1).

Example D

In vitro Enzyme Assays for Activity Specificity Determination

- 10 The ability of NAP of the present invention to act as a selective inhibitor of FXa catalytic activity or TF/VIIa activity was assessed by determining whether the test NAP would inhibit other enzymes in an assay at a concentration that was 100-fold higher than the concentration of the
- 15 following related serine proteases: thrombin, Factor Xa, Factor XIa, Factor XIIa, kallikrein, activated protein C, plasmin, recombinant tissue plasminogen activator (rt-PA), urokinase, chymotrypsin, and trypsin. These assays also are used to determine the specificity of NAPs having serine
- 20 protease inhibitory activity.

(1) General protocol for enzyme inhibition assays

- The buffer used for all assays was HBSA (Example A). All substrates were reconstituted in deionized water,
- 25 followed by dilution into HBSA prior to the assay. The amidolytic assay for determining the specificity of inhibition of serine proteases was conducted by combining in appropriate wells of a Corning microtiter plate, 50 μ l of HBSA, 50 μ l of NAP at a specified concentration diluted
- 30 in HBSA, or HBSA alone (uninhibited control velocity, V_o), and 50 μ l of a specified enzyme (see specific enzymes below). Following a 30 minute incubation at ambient temperature, 50 μ l of substrate were added to triplicate wells. The final concentration of reactants in a total
- 35 volume of 200 μ l of HBSA was: NAP (75 nM), enzyme (750 pM), and chromogenic substrate (as indicated below). The initial velocity of chromogenic substrate hydrolysis was measured as a change in absorbance at 405nm over a 5 minute period, in which less than 5% of the added substrate was
- 40 hydrolyzed. The velocities of test samples, containing NAP (V_i) were then expressed as a percent of the uninhibited

- 5 control velocity (V_o) by the following formula: $V_i/V_o \times 100$, for each of the enzymes.

(2) Specific enzyme assays

(a) Thrombin Assay

- 10 Thrombin catalytic activity was determined using the chromogenic substrate Pefachrome t-PA (CH_3SO_2 -D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm Ltd., Basel, Switzerland). The final concentration of Pefachrome t-PA was 250 μM (about 5-
15 times K_m). Purified human alpha-thrombin was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(b) Factor Xa Assay

- Factor Xa catalytic activity was determined using the
20 chromogenic substrate S-2765 (N-benzyloxycarbonyl-D-arginine-L-glycine-L-arginine-p-nitroaniline), obtained from Kabi Pharmacia Hepar, Inc. (Franklin, OH). All substrates were reconstituted in deionized water prior to use. The final concentration of S-2765 was 250 μM (about
25 5-times K_m). Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (FXa) was activated and prepared from Factor X as described [Bock, P.E., Craig, P.A., Olson, S.T., and Singh, P. Arch. Biochem. Biophys. 273:375-388 (1989)].

30

(c) Factor XIa Assay

- Factor FXIa catalytic activity was determined using the chromogenic substrate S-2366 (L-Pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia
35 Hepar, Franklin, OH). The final concentration of S-2366 was 750 μM . Purified human FXIa was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(d) Factor XIIa Assay

- 40 Factor FXIIa catalytic activity was determined using the chromogenic substrate Spectrozyme FXIIa (H-D-CHT-L-glycyl-L-arginine-p-nitroaniline), obtained from American

5 Diagnostica, Greenwich, CT). The final concentration of Spectrozyme FXIIa was 100 μ M. Purified human FXIIa was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

10 (e) Kallikrein Assay

Kallikrein catalytic activity was determined using the chromogenic substrate S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2302
15 was 400 μ M. Purified human kallikrein was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(f) Activated Protein C (aPC)

Activated Protein C catalytic activity was determined
20 using the chromogenic substrate Spectrozyme PCa (H-D-lysyl(-Cbo)-L-prolyl-L-arginine-p-nitroaniline) obtained from American Diagnostica Inc. (Greenwich, CT). The final concentration was 400 μ M (about 4 times K_m). Purified human aPC was obtained from Hematologic Technologies,
25 Inc. (Essex Junction, VT)

(g) Plasmin Assay

Plasmin catalytic activity was determined using the chromogenic substrate S-2366 (L-Pyroglutamyl-L-prolyl-L-
30 arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2366 was 300 μ M (about 4 times K_m). Purified human plasmin was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN)..
35

(h) Recombinant tissue plasminogen activator (rt-PA)

rt-PA catalytic activity was determined using the substrate, Pefachrome t-PA (CH₃SO₂-D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm
40 Ltd., Basel, Switzerland). The final concentration was 500 μ M (about 3 times K_m). Human rt-PA (Activase®) was obtained from Genentech, Inc. (So. San Fransisco, CA).

5

(i) Urokinase

Urokinase catalytic activity was determined using the substrate S-2444 (L-Pyroglutamyl-L-glycyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2444 was 150 μ M (about 7 times K_m). Human urokinase (Abbokinase®), purified from cultured human kidney cells, was obtained from Abbott Laboratories (North Chicago, IL).

15

(j) Chymotrypsin

Chymotrypsin catalytic activity was determined using the chromogenic substrate, S-2586 (Methoxy-succinyl-L-argininyl-L-prolyl-L-tyrosine-p-nitroaniline, which was obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2586 was 100 μ M (about 8 times K_m). Purified (3X-crystallized; CDI) bovine pancreatic-chymotrypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ).

25

(k) Trypsin

Trypsin catalytic activity was determined using the chromogenic substrate S-2222 (N-benzoyl-L-isoleucyl-L-glutamyl [-methyl ester]-L-arginine-p-nitroaniline, which was obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2222 was 300 μ M (about 5 times K_m). Purified human pancreatic trypsin was obtained from Scripps Laboratories (San Diego, CA).

Table 4 lists the inhibition of the amidolytic activity of FXa and 10 additional serine proteases by either recombinant AcaNAP-5 [SEQ. ID. NO. 4] or recombinant AcaNAP-6 [SEQ. ID. NO. 6] (both expressed in *Pichia pastoris*, as described), expressed as percent of control velocity. These NAPs demonstrate a high degree of specificity for the inhibition of FXa compared to the other, related serine proteases.

5

Table 4

Enzyme	% Control Velocity	% Control Velocity
	+ AcaNAP5	+AcaNAP6
FXa	1 \pm 1	14 \pm 1
FIIa	104 \pm 5	98 \pm 3
FXIa	34 \pm 12	98 \pm 3
FXIIa	103 \pm 6	100 \pm 4
kallikrein	102 \pm 4	101 \pm 3
aPC	95 \pm 2	98 \pm 1
plasmin	111 \pm 6	113 \pm 12
r-tPA	96 \pm 9	96 \pm 7
urokinase	101 \pm 14	96 \pm 2
chymotrypsin	105 \pm 0	100 \pm 11
trypsin	98 \pm 6	93 \pm 4

Table 5 lists the inhibitory effect of recombinant AcaNAPc2 [SEQ. ID. NO. 59] and recombinant AceNAP4 [SEQ. ID. NO. 62] (both expressed in *Pichia pastoris*, as described) on the amidolytic activity of 11 selected serine proteases. Inhibition is expressed as percent of control velocity. These data demonstrate that these NAPs possess a high degree of specificity for the serine proteases in Table 5.

5

Table 5

Enzyme	% Control Velocity	% Control Velocity
	+ AcaNAPc2	+ AceNAP4
FXa	84 ± 3	76 ± 3
FIIa	99 ± 3	93 ± 3
FXIa	103 ± 4	96 ± 1
FXIIa	97 ± 1	102 ± 2
kallikrein	101 ± 1	32 ± 1
aPC	97 ± 3	103 ± 1
plasmin	107 ± 9	100 ± 1
r-tPA	96 ± 2	108 ± 3
urokinase	97 ± 1	103 ± 4
chymotrypsin	99 ± 0	96 ± 4
trypsin	93 ± 4	98 ± 4

Example E10 Assays for measuring the inhibition of the fVIIa/TF complex by NAP(1) fVIIa/TF fIX activation assay

This Example measures the ability of NAPs of the present invention to act as an inhibitor of the catalytic complex of fVIIa/TF, which has a primary role in initiation of the coagulation response in the ex vivo prothrombin time assay (Example B). Activation of tritiated Factor IX by the rFVIIa/rTF/PLV complex was assessed by determining the respective intrinsic inhibition constant, K_i^* .

Lyophilized, purified, recombinant human factor VIIa was obtained from BiosPacific, Inc. (Emeryville, CA), and reconstituted in HBS (10 mM HEPES, pH 7.5, 150 mM sodium chloride) prior to use. Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (free FXa) was activated and

5 prepared from Factor X as described (Bock, P.E., Craig,
P.A., Olson, S.T., and Singh, P. Arch. Biochem. Biophys.
273:375-388 (1989)). Active site-blocked human Factor Xa
(EGR-FXa), which had been irreversibly inactivated with L-
10 Glutamyl-L-glycyl-L-arginyl chloromethylketone, was
obtained from Haematologic Technologies, Inc. (Essex
Junction, VT). Recombinant human tissue factor (rTF) was
produced by a baculovirus-expression system, and purified
to homogeneity by monoclonal antibody affinity
chromatography (Corvas International, Inc., San Diego,
15 CA).

The purified rTF apoprotein was incorporated into
phospholipid vesicles (rTF/PLV), consisting of
phosphotidyl choline (75%, w/v) and phosphotidyl serine
(25%, w/v) in the presence of detergent, as described by
20 Ruf et al. (Ruf, W., Miles, D.J., Rehemtulla, A., and
Edgington, T.S. Methods in Enzymology 222: 209-224
(1993)). The phospholipids were purchased from Avanti
Polar Lipids, (Alabaster, Alabama). The buffer used for
all assays was HBSA, HBS containing 0.1% (w/v) bovine
25 serum albumin. All reagents were obtained from Sigma
Chemical Co. (St. Louis, MO), unless otherwise indicated.

The activation of human ^3H -Factor IX (FIX) by the
rFVIIa/rTF complex was monitored by measuring the release
of the radiolabelled activation peptide. Purified human
30 FIX was obtained from Haematologic Technologies, Inc.
(Essex Junction, VT), and radioactively labelled by
reductive tritiation as described (Van Lenten & Ashwell,
1971, JBC 246, 1889-1894). The resulting tritiated
preparation of FIX had a specific activity of 194 clotting
35 units/mg as measured in immuno-depleted FIX deficient
plasma (Ortho), and retained 97% of its activity. The
radiospecific activity was 2.7×10^8 dpm/mg. The K_m for
the activation of ^3H -FIX by rFVIIa/rTF/PLV was 25 nM,
which was equivalent to the K_m obtained for untreated
40 (unlabelled) FIX.

The assay for K_i^* determinations was conducted as
follows: rFVIIa and rTF/PLV were combined in a

- 5 polypropylene test tube, and allowed to form a complex for 10 min in HBSA, containing 5 mM CaCl_2 . Aliquots of rFVIIa/rTF/PLV complex were combined in the appropriate polypropylene microcentrifuge tubes with EGR-FXa or free FXa, when included, and either the NAP test compound at 10 various concentrations, after dilution into HBSA, or HBSA alone (as V_0 (uninhibited velocity) control). Following an incubation of 60 min at ambient temperature, reactions were initiated by the addition of ^3H -FIX. The final concentration of the reactants in 420 μl of HBSA was:
- 15 rFVIIa [50 pM], rTF [2.7 nM], PLV [6.4 micromolar], either EGR-FXa or free FXa [300 pM], recombinant NAP [5-1,500 pM], ^3H -FIX [200 nM], and CaCl_2 [5mM]. In addition, a background control reaction was run that included all of the above reactants, except rFVIIa.
- 20 At specific time points (8, 16, 24, 32, and 40 min), 80 μl of the reaction mixture was added to an eppendorf tube that contained an equal volume of 50 mM EDTA in HBS with 0.5% BSA to stop the reaction; this was followed by the addition of 160 μL of 6% (w/v) trichloroacetic acid.
- 25 The protein was precipitated, and separated from the supernatant by centrifugation at 16,000Xg for 6 min at 4°C . The radioactivity contained in the resulting supernatant was measured by removing triplicate aliquots that were added to Scintiverse BD (Fisher Scientific,
- 30 Fairlawn, NJ), and quantitated by liquid scintillation counting. The control rate of activation was determined by linear regression analysis of the soluble counts released over time under steady-state conditions, where less than 5% of the tritiated FIX was consumed. The background control (<1.0% of control velocity) was 35 subtracted from all samples. Ratios of inhibited steady-state velocities (V_i), in the presence of a NAP, to the uninhibited control velocity of rFVIIa/TF alone (V_0) were plotted against the corresponding concentrations of NAP.
- 40 These data were then directly fit to an equation for tight-binding inhibitors [Morrison, J.F., and Walsh, C.T., Adv. Enzymol. 61:201-300 (1988)], from which the

5 apparent equilibrium dissociation inhibitory constant K_i^*
was calculated.

 The data for recombinant AcaNAP5, AcaNAP6, AcaNAPc2,
and AceNAP4 (prepared as described) is presented in Table
6 following Section B, below.

10

(2) Factor VIIa/Tissue factor amidolytic assay

 The ability of NAPs of the present invention to act
as an inhibitor of the amidolytic activity of the fVIIa/TF
complex was assessed by determining the respective
15 inhibition constant, K_i^* , in the presence and absence of
active site-blocked human Factor Xa (EGR-fXa).

 rFVIIa/rTF amidolytic activity was determined using
the chromogenic substrate S-2288 (H-D-isoleucyl-L-prolyl-
L-arginine-p-nitroaniline), obtained from Kabi Pharmacia
20 Hepar, Inc. (Franklin, OH). The substrate was
reconstituted in deionized water prior to use. rFVIIa and
rTF/PLV were combined in a polypropylene test tube, and
allowed to form a complex for 10 min in HBSA, containing
3 mM CaCl_2 . The assay for K_i^* determinations was
25 conducted by combining in appropriate wells of a Corning
microtiter plate 50 μL of the rFVIIa/rTF/PLV complex, 50
 μL of EGR-FXa, and 50 μL of either the NAP test compound
at various concentrations, after dilution into HBSA, or
HBSA alone (for V_o (uninhibited velocity) measurement).
30 Following an incubation of 30 min at ambient temperature,
the triplicate reactions were initiated by adding 50 μL of
S-2288. The final concentration of reactants in a total
volume of 200 μL of HBSA was: recombinant NAP (.025-25
nM), rFVIIa (750 pM), rTF (3.0 nM), PLV (6.4 micromolar),
35 EGR-FXa (2.5 nM), and S-2288 (3.0 mM, 3X K_m).

 The amidolytic activity of rFVIIa/rTF/PLV was
measured as a linear increase in the absorbance at 405 nm
over 10 min (velocity), using a Thermo Max[®] Kinetic
Microplate Reader (Molecular Devices, Palo Alto, CA),
40 under steady-state conditions, where less than 5% of the
substrate was consumed. Ratios of inhibited pre-
equilibrium, steady-state velocities (V_i), in the presence

5 of NAP, to the uninhibited velocity in the presence of
 free fXa alone (V_0) were plotted against the corresponding
 concentrations of NAP. These data were then directly fit
 to the same equation for tight-binding inhibitors, used
 in Example E.1., from which the apparent equilibrium
 10 dissociation inhibitory constant K_i^* was calculated.

Table 6 below gives the K_i^* values of recombinant
 AcaNAPc2 [SEQ. ID. NO. 59], AceNAP4 [SEQ. ID. NO. 62],
 AcaNAP5 [SEQ. ID. NO. 4], and AcaNAP6 [SEQ. ID. NO. 6]
 (prepared in *Pichia pastoris*, as described) in inhibitory
 15 assays of rFVIIa/rTF activity. The data shows the utility
 of AcaNAPc2 and AceNAP4 as potent inhibitors of the human
 rFVIIa/rTF/PLV complex in the absence and presence of
 either free FXa or active site-blocked FXa. The *in vitro*
 activity of AcaNAPc2P (see Example 17) was substantially
 20 the same as AcaNAPc2.

Table 6

NAP Compound	K_i^* (pM)				
	Amidolytic Assay		^3H -FIX Activation		
	No FXa Addition	Plus EGR- FXa	No FXa Addition	+ free FXa	+ EGR-FXa
AcaNAPc2	NI	36 ± 20	NI	35 ± 5	8.4 ± 1.5
AceNAP4	$59,230 \pm$ $3,600$	378 ± 37	ND	ND	ND
AcaNAP5	NI	NI	NI	NI	NI
AcaNAP6	NI	NI	NI	NI	NI

25 NI=no inhibition
 ND=not determined

5 Example FIn vivo Models of NAP activity(1) Evaluation of the antithrombotic activity of NAP in the rat model of FeCl₃-induced platelet-dependent arterial thrombosis

10 The antithrombotic (prevention of thrombus formation) properties of NAP were evaluated using the established experimental rat model of acute vascular thrombosis.

The rat FeCl₃ model is a well characterized model of platelet dependent, arterial thrombosis which has been
15 used to evaluate potential antithrombotic compounds. Kurz, K. D., Main, B. W., and Sandusky, G. E., *Thromb. Res.*, 60: 269-280 (1990). In this model a platelet-rich, occlusive thrombus is formed in a segment of the rat carotid artery treated locally with a fresh solution of FeCl₃ absorbed to
20 a piece of filter paper. The FeCl₃ is thought to diffuse into the treated segment of artery and cause de-endothelialization of the affected vessel surface. This results in the exposure of blood to subendothelial structures which in turn cause platelet adherence,
25 thrombin formation and platelet aggregation. The net result is occlusive thrombus formation. The effect of a test compound on the incidence of occlusive thrombus formation following application of FeCl₃ is monitored by ultrasonic flowtometry and is used as the primary end
30 point. The use of flowtometry to measure carotid artery blood flow, is a modification of the original procedure in which thermal detection of clot formation was employed. Kurz, K. D., Main, B. W., and Sandusky, G. E., *Thromb. Res.*, 60: 269-280 (1990).

35

(a) Intravenous administration

Male Harlan Sprague Dawley rats (420-450 g) were acclimated at least 72 hours prior to use and fasted for 12 hours prior to surgery with free access to water. The
40 animals were prepared, anesthetized with Nembutal followed by the insertion of catheters for blood pressure monitoring, drug and anesthesia delivery. The left

5 carotid artery was isolated by making a midline cervical
incision followed by blunt dissection and spreading
techniques to separate a 2 cm segment of the vessel from
the carotid sheath. A silk suture is inserted under the
proximal and distal ends of the isolated vessel to provide
10 clearance for the placement of a ultrasonic flow probe
(Transonic) around the proximal end of the vessel. The
probe is then secured with a stationary arm.

Following surgery the animals were randomized in
either a control (saline) or treatment (recombinant
15 AcaNAP5) group. The test compound (prepared in P. pastoris
according to Example 3) was administered as a single
intravenous bolus at the doses outlined in Table 7 after
placement of the flow probe and 5 min prior to the
thrombogenic stimulus. At t=0, a 3mm diameter piece of
20 filter paper (Whatman #3) soaked with 10 μ L of a 35%
solution of fresh FeCl₃ (made up in water) was applied to
the segment of isolated carotid artery distal to the flow
probe. Blood pressure, blood flow, heart rate, and
respiration were monitored for 60 minutes. The incidence
25 of occlusion (defined as the attainment of zero blood
flow) was recorded as the primary end point.

The efficacy of AcaNAP5 [SEQ. ID. NO. 4] as an
antithrombotic agent in preventing thrombus formation in
this in vivo model was demonstrated by the dose-dependent
30 reduction in the incidence of thrombotic occlusion, as
shown in Table 7 below.

5

Table 7

Treatment Group	Dose (mg/kg)	n	Incidence of Occlusion
Saline	-----	8	8/8
AcaNAP5	0.001	8	7/8
AcaNAP5	0.003	8	5/8
AcaNAP5	0.01	8	3/8*
AcaNAP5	0.03	8	1/8*
AcaNAP5	0.1	8	0/8*
AcaNAP5	0.3	4	0/4*
AcaNAP5	1.0	2	0/2*

*-p≤0.05 from saline control by Fishers test

- 10 The effective dose which prevents 50% of thrombotic occlusions in this model (ED₅₀) can be determined from the above data by plotting the incidence of occlusion versus the dose administered. This allows a direct comparison of the antithrombotic efficacy of AcaNAP5 with other
- 15 antithrombotic agents which have also been evaluated in this model as described above. Table 8 below lists the ED₅₀ values for several well known anticoagulant agents in this model compared to AcaNAP5.

5

Table 8

Compound	ED ₅₀ ^a
Standard Heparin	300 U/kg
Argatroban	3.8 mg/kg
Hirulog TM	3.0 mg/kg
rTAP ^b	0.6 mg/kg
AcaNAP5	0.0055 mg/kg

^aED₅₀ is defined as the dose that prevents the incidence of complete thrombotic occlusion in 50% of animals tested

10 ^b-recombinant Tick Anticoagulant Peptide, Vlasuk et al. Thromb. Haemostas. 70: 212-216 (1993)

(b) Subcutaneous administration

The antithrombotic effect of AcaNAP5 compared to
15 Low Molecular Weight heparin (Enoxaparin; Lovenox, Rhone-Poulenc Rorer) after subcutaneous administration was evaluated in rats using the FeCl₃ model. The model was performed in an identical manner to that described above with the exception that the compound was administered
20 subcutaneously and efficacy was determined at two different times: 30 and 150 minutes after administration. To accomplish this, both carotid arteries were employed in a sequential manner. The results of these experiments indicate that AcaNAP5 [SEQ. ID. NO. 4] is an effective
25 antithrombotic agent in vivo after subcutaneous administration. The results are shown below in Table 9.

Table 9

Compound	30" ED ₅₀ ^a (mg/kg)	150" ED ₅₀ ^a (mg/kg)
Low Molecular Weight Heparin	30.0	15.0
AcaNAP5	0.07	0.015

30

^aED₅₀ is defined as the dose that prevents the incidence of complete thrombotic occlusion in 50% of animals tested.

5 (2) Deep Wound Bleeding Measurement

 A model of deep wound bleeding was used to measure the effect of NAP on bleeding and compare the effect with that of Low Molecular Weight Heparin.

 Male rats were anesthetized and instrumented in an
10 identical manner to those undergoing the FeCl₃ model.
 However, FeCl₃ was not applied to the carotid artery. The deep surgical wound in the neck that exposes the carotid artery was employed to quantify blood loss over time. Blood loss was measured over a period of 3.5 hours
15 following subcutaneous administration of either AcaNAP5 or LMWH. The wound was packed with surgical sponges which were removed every 30 minutes. The sponges were subsequently immersed in Drabkin's reagent (sigma Chemical Co., St. Louis, MO) which lyses the red blood cells and
20 reacts with hemoglobin in a colorimetric fashion. The colorimetric samples were then quantified by measuring absorbance at 550 nM, which provides a determination of the amount of blood in the sponge.

 The dose response characteristics for both test
25 compounds are shown in Figure 15 along with efficacy data for both compounds. AcaNAP5 [SEQ. ID. NO. 4] was much more potent than Low Molecular Weight heparin in preventing occlusive arterial thrombus formation in this model. Furthermore, animals treated with NAP bled less
30 than those treated with Low Molecular Weight heparin.

 The data presented in Tables 7 and 9 and Figure 15 clearly demonstrate the effectiveness of NAP in preventing occlusive thrombus formation in this experimental model. The relevance of this data to preventing human thrombosis
35 is clear when compared to the other anticoagulant agents, listed in Table 8. These agents were been evaluated in the same experimental models described therein, in an identical manner to that described for NAPs, and in this experimental model and have demonstrated antithrombotic
40 efficacy in preventing thrombus formation clinically, as described in the following literature citations: Heparin-Hirsh, J. N. Engl. J. Med 324:1565-1574 1992, Cairns, J.A.

5 et al. Chest 102: 456S-481S (1992); Argatroban-Gold, H.K.
et al. J. Am. Coll. Cardiol. 21: 1039-1047 (1993); and
Hirulog™-Sharma, G.V.R.K. et al. Am. J. Cardiol. 72:
1357-1360 (1993) and Lidón, R.M. et al.. Circulation 88:
1495-1501 (1993).

10

Example G.

Pig Model Of Acute Coronary Artery Thrombosis

The protocol used in these studies is a modification
of a thrombosis model which has been reported previously
15 (Lucchesi, B.R., et al., (1994), *Brit. J. Pharmacol.*
113:1333-1343).

Animals were anesthetized and instrumented with
arterial and venous catheters (left common carotid and
external jugular, respectively). A thoracotomy was made
20 in the 4th intercostal space and the heart was exposed.
The left anterior descending (LAD) coronary artery was
isolated from the overlying connective tissue and was
instrumented with a Doppler flow probe and a 17 gauge
ligature stenosis. An anodal electrode also was implanted
25 inside the vessel.

Baseline measurements were taken and the NAP or
placebo to be tested was administered via the external
jugular vein. Five minutes after administration, a direct
current (300 μ A, DC) was applied to the stimulating
30 electrode to initiate intimal damage to the coronary
endothelium and begin thrombus formation. Current
continued for a period of 3 hours. Animals were observed
until either 1 hour after the cessation of current or the
death of the animal, whichever came first.

35 Table 10 presents data demonstrating the incidence of
occlusion in animals administered AcaNAP5 or AcaNAPc2P
(see Example 17) at three increasing doses of NAP. The
incidence of occlusion in the animals receiving placebo
was 8/8 (100%). Time to occlusion in placebo treated
40 animals was 66.6 ± 7.5 min. (mean \pm sem). Vessels in
AcaNAP treated pigs that failed to occlude during the 4
hour period of observation were assigned an arbitrary time

5 to occlusion of 240 minutes in order to facilitate statistical comparisons.

The data demonstrate AcaNAP5 and AcaNAPc2P were similarly efficacious in this setting; both prolonged the time to coronary artery occlusion in a dose dependent
 10 manner. Furthermore, both molecules significantly prolonged in time to occlusion at a dose (0.03 mg/kg i.v.) that did not produce significant elevations in bleeding. These data, and other, suggest AcaNAP5 and AcaNAPc2P have favorable therapeutic indices.

15

Table 10. Comparision of primary endpoints between AcaNAPc2P and AcaNAP5 after intravenous dosing in the pig model of acute coronary artery thrombosis.

20

Dose (i.v.) (mg/kg)	Incidence of Occlusion		Time of Occlusion (min)		Total Blood Loss (ml)	
	AcaNAP5	AcaNAPc2P	AcaNAP5	AcaNAPc2P	AcaNAP5	AcaNAPc2P
0.01	6/6	6/6	107 ± 13.0	105 ± 6.2	2.8 ± 0.8	1.6 ± 0.3
0.03	5/6	4/6	150 ± 23.2	159 ± 27	5.6 ± 1.4	4.9 ± 1.4
0.10	4/6	2/6†	187 ± 22.9*	215 ± 25*	43.5 ± 18*	17.6 ± 7.9*

† p<0.05 vs saline (8/8), Fisher's Exact; *p<0.05 vs saline, ANOVA, Dunnett's multiple comparison test.

5 Claims

1. An isolated protein having anticoagulant activity and having one or more NAP domains, wherein each NAP domain includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
10 Cys-A9-Cys-A10 [FORMULA II], wherein

(a) A1 is an amino acid sequence of 7 to 8 amino acid residues;

(b) A2 is an amino acid sequence;

(c) A3 is an amino acid sequence of 3 amino acid
15 residues;

(d) A4 is an amino acid sequence;

(e) A5 is an amino acid sequence of 3 to 4 amino acid residues;

(f) A6 is an amino acid sequence;

20 (g) A7 is an amino acid residue;

(h) A8 is an amino acid sequence of 11 to 12 amino acid residues;

(i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and

25 (j) A10 is an amino acid sequence;

wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid
30 residues.

2. The protein of claim 1, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues.

35

3. The protein of claim 1, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg.
40

- 5 4. The protein of claim 3, wherein A3 is selected
from the group consisting of
 Glu-Ala-Lys,
 Glu-Arg-Lys,
 Glu-Pro-Lys,
10 Glu-Lys-Lys,
 Glu-Ile-Thr,
 Glu-His-Arg,
 Glu-Leu-Lys, and
 Glu-Thr-Lys.
- 15 5. The protein of claim 1, wherein A4 is an amino
acid sequence having a net anionic charge.
6. The protein of claim 1, wherein A7 is Val.
- 20 7. The protein of claim 1, wherein A7 is Ile.
8. The protein of claim 1, wherein A8 includes the
amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID.
25 NO. 68], wherein
 (a) A8_a is the first amino acid residue in A8,
 (b) at least one of A8_a and A8_b is selected from the
group consisting of Glu or Asp, and
 (c) A8_c through A8_g are independently selected amino
30 acid residues.
9. The protein of claim 8, wherein
 (a) A8_a is Glu or Asp,
 (b) A8_b is an independently selected amino acid
35 residue,
 (c) A8_c is Gly,
 (d) A8_d is selected from the group consisting of
Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
40 (f) A8_f is Arg, and
 (g) A8_g is selected from Asp and Asn.

- 5 10. The protein of claim 9, wherein A8_c-A8_d-A8_e-A8_f-
A8_g is selected from the group consisting of
Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
10 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].
11. The protein of claim 8, wherein
(a) A8_a is an independently selected amino acid
15 residue,
(b) A8_b is Glu or Asp,
(c) A8_c is Gly,
(d) A8_d is selected from the group consisting of
Phe, Tyr, and Leu,
20 (e) A8_e is Tyr,
(f) A8_f is Arg, and
(g) A8_g is selected from Asp and Asn.
12. The protein of claim 11, wherein A8_c-A8_d-A8_e-
25 A8_f-A8_g is selected from the group consisting of
Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
30 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].
13. The protein of claim 8, wherein A8_c-A8_d-A8_e-A8_f-
A8_g is selected from the group consisting of
Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
35 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].
- 40 14. The protein of claim 1, wherein A10 includes an
amino acid sequence selected from the group consisting of
Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],

5 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

15 15. The protein of claim 14, wherein A10 includes
the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO.
74].

16. The protein of claim 15 having a NAP domain with
an amino acid sequence substantially the same as that of
15 AcaNAP5 [SEQ. ID. NO. 40] or AcaNAP6 [SEQ. ID. NO. 41].

17. The protein of claim 14, wherein A10 includes
the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO.
75].

20

18. The protein of claim 14, wherein A10 includes
the amino acid sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID.
NO. 76].

25 19. The protein of claim 14, wherein A10 includes
the amino acid sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO.
77].

30 20. The protein of claim 1 derived from a nematode
species.

21. The protein of claim 20, wherein said nematode
species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
35 *duodenale*, *Necator americanus*, and *Heligomosomoides*
polygyrus.

22. The protein of claim 1, wherein
(a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and
40 A3_b are independently selected amino acid residues;
(b) A4 is an amino acid sequence having a net
anionic charge;

- 5 (c) A7 is selected from the group consisting of Val
and Ile;
- (d) A8 includes an amino acid sequence selected from
the group consisting of
- 10 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
- (e) A10 includes an amino sequence selected from the
15 group consisting of
- Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].
- 20
23. The protein of claim 22 having a NAP domain
substantially the same as NAP domains selected from
AcaNAP5 [SEQ. ID. NO. 40] and AcaNAP6 [SEQ. ID. NO. 41].
- 25 24. The protein of claim 22 derived from a nematode
species.
25. The protein of claim 24, wherein said nematode
species is selected from the group consisting of
- 30 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
polygyrus.
26. The protein of claim 1, wherein
- 35 (a) A3 is selected from the group consisting of
- Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,
Glu-Lys-Lys,
40 Glu-Ile-Thr,
Glu-His-Arg,
Glu-Leu-Lys, and

- 5 Glu-Thr-Lys;
- (b) A4 is an amino acid sequence having a net
 anionic charge;
- (c) A7 is Val or Ile;
- (d) A8 includes an amino acid sequence selected from
10 the group consisting of
 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78],
 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81],
15 and
 A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82],
 wherein at least one of A8_a and A8_b is Glu or Asp;
- (e) A9 is an amino acid sequence of five amino acid
 residues; and
- 20 (f) A10 includes an amino acid sequence selected
 from the group consisting of
 Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
25 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

27. The protein of claim 26 having a NAP domain
substantially the same as NAP domains selected from
AcaNAP5 [SEQ. ID. NO. 40] and AcaNAP6 [SEQ. ID. NO. 41].
30
28. The protein of claim 26 derived from a nematode
species.
29. The protein of claim 28, wherein said nematode
35 species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
polygyrus.
- 40 30. An isolated protein having Factor Xa inhibitory
activity selected from the group consisting of AcaNAP5
[SEQ. ID. NO. 40] and AcaNAP6 [SEQ. ID. NO. 41].

5

31. An isolated recombinant cDNA molecule encoding a protein having Factor Xa inhibitory activity and having one or more NAP domains, wherein each NAP domain includes the sequence:

10 Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 [FORMULA II], wherein

(a) A1 is an amino acid sequence of 7 to 8 amino acid residues;

(b) A2 is an amino acid sequence;

15 (c) A3 is an amino acid sequence of 3 amino acid residues;

(d) A4 is an amino acid sequence;

(e) A5 is an amino acid sequence of 3 to 4 amino acid residues;

20 (f) A6 is an amino acid sequence;

(g) A7 is an amino acid residue;

(h) A8 is an amino acid sequence of 11 to 12 amino acid residues;

(i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and

(j) A10 is an amino acid sequence;

wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP
30 domain has in total less than about 120 amino acid residues.

32. The cDNA molecule of claim 31, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are
35 independently selected amino acid residues.

33. The cDNA molecule of claim 31, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and
40 Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg.

- 5 34. The cDNA molecule of claim 33, wherein A3 is
selected from the group consisting of
Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,
10 Glu-Lys-Lys,
Glu-Ile-Thr,
Glu-His-Arg,
Glu-Leu-Lys, and
Glu-Thr-Lys.
- 15 35. The cDNA molecule of claim 31, wherein A4 is an
amino acid sequence having a net anionic charge.
- 20 36. The cDNA molecule of claim 31, wherein A7 is
Val.
37. The cDNA molecule of claim 31, wherein A7 is
Ile.
- 25 38. The cDNA molecule of claim 31, wherein A8
includes an amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-
A8_g [SEQ. ID. NO. 68], wherein
 (a) A8_a is the first amino acid residue in A8,
 (b) at least one of A8_a and A8_b is selected from the
30 group consisting of Glu or Asp, and
 (c) A8_c through A8_g are independently selected amino
acid residues.
- 35 39. The cDNA molecule of claim 38, wherein
 (a) A8_a is Glu or Asp,
 (b) A8_b is an independently selected amino acid
residue,
 (c) A8_c is Gly,
 (d) A8_d is selected from the group consisting of
40 Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
 (f) A8_f is Arg, and

5 (g) A8_g is selected from Asp and Asn.

40. The cDNA molecule of claim 39, wherein A8_c-A8_d-
A8_e-A8_f-A8_g is selected from the group consisting of

10 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

15 41. The cDNA molecule of claim 38, wherein

(a) A8_a is an independently selected amino acid
residue,

(b) A8_b is Glu or Asp,

(c) A8_c is Gly,

20 (d) A8_d is selected from the group consisting of
Phe, Tyr, and Leu,

(e) A8_e is Tyr,

(f) A8_f is Arg, and

(g) A8_g is selected from Asp and Asn.

25

42. The cDNA molecule of claim 41, wherein A8_c-A8_d-
A8_e-A8_f-A8_g is selected from the group consisting of

30 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

43. The cDNA molecule of claim 38, wherein A8_c-A8_d-
35 A8_e-A8_f-A8_g is selected from the group consisting of

Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
40 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

- 5 44. The cDNA molecule of claim 31, wherein A10
includes an amino acid sequence selected from the group
consisting of
 Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
10 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].
45. The cDNA molecule of claim 44, wherein A10
includes the amino acid sequence Glu-Ile-Ile-His-Val [SEQ.
15 ID. NO. 74].
46. The cDNA molecule of claim 45 having a
nucleotide sequence substantially the same as that coding
for AcaNAP5 [SEQ. ID. NO. 3] or AcaNAP6 [SEQ. ID. NO. 5].
20
47. The cDNA molecule of claim 44, wherein A10
includes the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ.
ID. NO. 75].
- 25 48. The cDNA molecule of claim 44, wherein A10
includes the amino acid sequence Phe-Ile-Thr-Phe-Ala-Pro
[SEQ. ID. NO. 76].
49. The cDNA molecule of claim 44, wherein A10
30 includes the amino acid sequence Met-Glu-Ile-Ile-Thr [SEQ.
ID. NO. 77].
50. The cDNA molecule of claim 31 derived from a
nematode species.
35
51. The cDNA molecule of claim 50, wherein said
nematode species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
40 *polygyrus*.
52. The cDNA molecule of claim 31, wherein

- 5 (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and
A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net
anionic charge;
- (c) A7 is selected from the group consisting of Val
10 and Ile;
- (d) A8 includes an amino acid sequence selected from
the group consisting of
- 15 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
- (e) A10 includes an amino sequence selected from the
group consisting of
- 20 Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].
- 25 53. The cDNA of claim 52 that is selected from cDNAs
substantially the same as cDNAs coding for AcaNAP5 [SEQ.
ID. NO. 3] and AcaNAP6 [SEQ. ID. NO. 5].
54. The cDNA molecule of claim 52 derived from a
30 nematode species.
55. The cDNA molecule of claim 54, wherein said
nematode species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
35 *duodenale*, *Necator americanus*, and *Heligomosomoides*
polygyrus.
56. The cDNA molecule of claim 31, wherein
- (a) A3 is selected from the group consisting of
- 40 Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,

- 5 Glu-Lys-Lys,
 Glu-Ile-Thr,
 Glu-His-Arg,
 Glu-Leu-Lys, and
 Glu-Thr-Lys;
- 10 (b) A4 is an amino acid sequence having a net
 anionic charge;
 (c) A7 is Val or Ile;
 (d) A8 includes an amino acid sequence selected from
 the group consisting of
- 15 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78],
 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81],
 and
- 20 A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82],
 wherein at least one of A8_a and A8_b is Glu or Asp;
 (e) A9 is an amino acid sequence of five amino acid
 residues; and
 (f) A10 includes an amino acid sequence selected
- 25 from the group consisting of
 Glu-Ile-Ile-His-Val, [SEQ. ID. NO. 74]
 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

30

57. The cDNA molecule of claim 56 that is selected from cDNAs coding for a NAP domain substantially the same as NAP domains selected from AcaNAP5 [SEQ. ID. NO. 40] and AcaNAP6 [SEQ. ID. NO. 41].

35

58. The cDNA molecule of claim 56 derived from a nematode species.

59. The cDNA molecule of claim 58, wherein said
40 nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides*

5 *polygyrus*.

60. A cDNA molecule encoding a protein having Factor Xa inhibitory activity selected from the group consisting of proteins having NAP domains substantially the same as
10 AcaNAP5 [SEQ. ID. NO. 40] or AcaNAP6 [SEQ. ID. NO. 41].

61. A pharmaceutical composition comprising the protein of claim 1.

15 62. A pharmaceutical composition comprising the protein of claim 22.

63. A pharmaceutical composition comprising the protein of claim 26.

20

64. A pharmaceutical composition comprising a protein selected from the group consisting of AcaNAP5 [SEQ. ID. NO. 40] and AcaNAP6 [SEQ. ID. NO. 41].

25 65. A method of inhibiting blood coagulation comprising administering a protein of claim 1 with a pharmaceutically acceptable carrier.

66. A method of inhibiting blood coagulation
30 comprising administering a protein of claim 22 with a pharmaceutically acceptable carrier.

67. A method of inhibiting blood coagulation comprising administering a protein of claim 26 with a
35 pharmaceutically acceptable carrier.

68. A method of inhibiting blood coagulation comprising administering a protein selected from the group consisting of AcaNAP5 [SEQ. ID. NO. 40] and AcaNAP6 [SEQ.
40 ID. NO. 41].

69. A protein of claim 1, wherein said protein has

5 two NAP domains.

70. A protein of claim 22, wherein said protein has two NAP domains.

10 71. A protein of claim 26, wherein said protein has two NAP domains.

72. A protein of claim 1 wherein said NAP domain includes the amino acid sequence:

15 Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10

wherein

(a) Cys-A1 is selected from SEQ. ID NOS. 67 and 156;

(b) Cys-A2-Cys is selected from one of SEQ. ID. NOS.
20 157 to 159;

(c) A3-Cys-A4 is selected from one of SEQ. ID. NOS.
160 to 173.

(d) Cys-A5 is selected from SEQ. ID. NOS. 174 and
175;

(e) Cys-A6 is selected from one of SEQ. ID. NOS. 176
25 to 178;

(f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 179
and 180;

(g) Cys-A9 is selected from one of SEQ. ID. NOS. 181
30 to 183; and

(h) Cys-A10 is selected from one of SEQ. ID. NOS.
184 to 204.

73. An isolated protein having anticoagulant activity and having one or more NAP domains, wherein each
35 NAP domain includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 (FORMULA III),

wherein

(a) A1 is an amino acid sequence of 7 to 8 amino
40 acid residues;

(b) A2 is an amino acid sequence;

(c) A3 is an amino acid sequence of 3 amino acid

- 5 residues;
- (d) A4 is an amino acid sequence;
 - (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
 - (f) A6 is an amino acid sequence;
 - 10 (g) A7 is an amino acid residue;
 - (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
 - (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
 - 15 (j) A10 is an amino acid sequence;
- wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid
- 20 residues.

74. The protein of claim 73, wherein A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues.

25 75. The protein of claim 73, wherein A3 is Asp-Lys-Lys.

76. The protein of claim 73, wherein A4 is an amino acid sequence having a net anionic charge.

30

77. The protein of claim 73, wherein A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 85], wherein A5_a through A5_d are independently selected amino acid

35 residues.

78. The protein of claim 77, wherein A5_a is Leu and A5_c is Arg.

40 79. The protein of claim 73, wherein A7 is selected from the group consisting of Val and Ile.

5 80. The protein of claim 73, wherein A7 is Val.

81. The protein of claim 73, wherein A8 includes an amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

- 10 (a) A8_a is the first amino acid residue in A8,
 (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
 (c) A8_c through A8_g are independently selected amino acid residues.

15

82. The protein of claim 81, wherein

- (a) A8_a is Glu or Asp,
 (b) A8_b is an independently selected amino acid residue,
20 (c) A8_c is Gly,
 (d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
 (f) A8_f is Arg, and
25 (g) A8_g is selected from Asp and Asn.

83. The protein of claim 82, wherein A8_c-A8_d-A8_e-A8_f-A8_g is Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70].

30 84. The protein of claim 81, wherein

- (a) A8_a is an independently selected amino acid residue,
 (b) A8_b is Glu or Asp,
 (c) A8_c is Gly,
35 (d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
 (f) A8_f is Arg, and
 (g) A8_g is selected from Asp and Asn.

40

85. The protein of claim 84, wherein A8_c-A8_d-A8_e-A8_f-A8_g is Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70].

5

86. The protein of claim 73 derived from a nematode species.

87. The protein of claim 86, wherein said nematode
10 species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

15 88. The protein of claim 73, wherein
(a) A3 is has the sequence Asp-A3_a-A3_b, wherein A3_a
and A3_b are independently selected amino acid residues;
(b) A4 is an amino acid sequence having a net
anionic charge;
20 (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID.
NO. 85], wherein A5_a through A5_d are independently
selected amino acid residues, and
(d) A7 is selected from the group consisting of Val
and Ile.

25

89. The protein of claim 88 having a NAP domain with
an amino acid sequence substantially the same as the NAP
domain of AcaNAPc2 [SEQ. ID. NO. 59].

30 90. The protein of claim 88 derived from a nematode
species.

91. The protein of claim 90, wherein said nematode
species is selected from the group consisting of
35 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

92. The protein of claim 73, wherein
40 (a) A3 is Asp-Lys-Lys;
(b) A4 is an amino acid sequence having a net
anionic charge;

- 5 (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d, wherein A5_a is Leu, A5_c is Arg, and A5_b and A5_d are independently selected amino acid residues [SEQ. ID. NO. 357],
(d) A7 is Val; and
(e) A8 includes an amino acid sequence A8_a-A8_b-Gly-
10 Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], wherein at least one of A8_a and A8_b is Glu or Asp.

93. The protein of claim 92 having a NAP domain with an amino acid sequence substantially the same as the NAP
15 domain of AcaNAPc2 [SEQ. ID. NO. 59].

94. The protein of claim 92 derived from a nematode species.

20 95. The protein of claim 94, wherein said nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

25 96. An isolated protein having Factor VIIa/TF inhibitory activity having a NAP domain with an amino acid sequence that is substantially the same as the NAP domain of AcaNAPc2 [SEQ. ID. NO. 59].

30 97. An isolated recombinant cDNA molecule encoding a protein having anticoagulant activity and having one or more NAP domains, wherein each NAP domain includes the sequence:

35 Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 [FORMULA III], wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
(b) A2 is an amino acid sequence;
40 (c) A3 is an amino acid sequence of 3 amino acid residues;
(d) A4 is an amino acid sequence;

- 5 (e) A5 is an amino acid sequence of 3 to 4 amino
acid residues;
 (f) A6 is an amino acid sequence;
 (g) A7 is an amino acid residue;
 (h) A8 is an amino acid sequence of 11 to 12 amino
10 acid residues;
 (i) A9 is an amino acid sequence of 5 to 7 amino
acid residues; and
 (j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently
15 selected number of independently selected amino acid
residues and each sequence is selected such that each NAP
domain has in total less than about 120 amino acid
residues.
- 20 98. The cDNA molecule of claim 97, wherein A3 has
the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are
independently selected amino acid residues.
99. The cDNA molecule of claim 97, wherein A3 is
25 Asp-Lys-Lys.
100. The cDNA molecule of claim 97, wherein A4 is an
amino acid sequence having a net anionic charge.
- 30 101. The cDNA molecule of claim 97, wherein A5 has
the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 85], wherein
A5_a through A5_d are independently selected single amino
acid residues.
- 35 102. The cDNA molecule of claim 101, wherein A5_a is
Leu and A5_c is Arg.
103. The cDNA molecule of claim 97, wherein A7 is
selected from the group consisting of Val and Ile.
- 40 104. The cDNA molecule of claim 97, wherein A7 is
Val.

5

105. The cDNA molecule of claim 97, wherein A8 includes an amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

- (a) A8_a is the first amino acid residue in A8,
- 10 (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
- (c) A8_c through A8_g are independently selected amino acid residues.

15

106. The cDNA molecule of claim 105, wherein

- (a) A8_a is Glu or Asp,
- (b) A8_b is an independently selected amino acid residue,
- (c) A8_c is Gly,
- 20 (d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,
- (e) A8_e is Tyr,
- (f) A8_f is Arg, and
- (g) A8_g is selected from Asp and Asn.

25

107. The cDNA molecule of claim 106, wherein A8_c-A8_d-A8_e-A8_f-A8_g is Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70].

108. The cDNA molecule of claim 105, wherein

- 30 (a) A8_a is an independently selected amino acid residue,
- (b) A8_b is Glu or Asp,
- (c) A8_c is Gly,
- (d) A8_d is selected from the group consisting of
- 35 Phe, Tyr, and Leu,
- (e) A8_e is Tyr,
- (f) A8_f is Arg, and
- (g) A8_g is selected from Asp and Asn.

40

109. The cDNA molecule of claim 108, wherein A8_c-A8_d-A8_e-A8_f-A8_g is Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70].

5 110. The cDNA molecule of claim 97 derived from a nematode species.

 111. The cDNA molecule of claim 110, wherein said nematode species is selected from the group consisting of
10 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

 112. The cDNA molecule of claim 97, wherein
15 (a) A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
 (b) A4 is an amino acid sequence having a net anionic charge;
 (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d, wherein A5_a
20 through A5_d are independently selected amino acid residues [SEQ. ID. NO. 85], and
 (d) A7 is selected from the group consisting of Val and Ile.

25 113. The cDNA molecule of claim 112 having a nucleotide sequence coding for an amino acid sequence substantially the same as the NAP domain of AcaNAPc2 [SEQ. ID. NO. 59].

30 114. The cDNA molecule of claim 112 derived from a nematode species.

 115. The cDNA molecule of claim 114, wherein said nematode species is selected from the group consisting of
35 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

 116. The cDNA molecule of claim 97, wherein
40 (a) A3 is Asp-Lys-Lys;
 (b) A4 is an amino acid sequence having a net anionic charge;

- 5 (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 129], wherein A5_a is Leu, A5_c is Arg, and A5_b and A5_d are independently selected amino acid residues,
- (d) A7 is Val; and
- (e) A8 includes an amino acid sequence A8_a-A8_b-Gly-
- 10 Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], wherein at least one of A8_a and A8_b is Glu or Asp.

 117. The cDNA molecule of claim 116 having a nucleotide sequence which codes for an amino acid sequence

15 substantially the same as AcaNAPc2 [SEQ. ID. NO. 59].

 118. The cDNA molecule of claim 116 derived from a nematode species.

20 119. The cDNA molecule of claim 118, wherein said nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

25 120. An isolated cDNA molecule encoding a protein having Factor VIIa/TF inhibitory activity and a NAP domain with an amino acid sequence that is substantially the same as the NAP domain of AcaNAPc2 [SEQ. ID. NO. 59].

30 121. A pharmaceutical composition comprising the protein of claim 73.

 122. A pharmaceutical composition comprising the

35 protein of claim 88.

 123. A pharmaceutical composition comprising the protein of claim 92.

40 124. A pharmaceutical composition comprising an AcaNAPc2 protein [SEQ. ID. NO. 59].

5 125. A method of inhibiting blood coagulation
comprising administering a protein of claim 73 with a
pharmaceutically acceptable carrier.

10 126. A method of inhibiting blood coagulation
comprising administering a protein of claim 88 with a
pharmaceutically acceptable carrier.

15 127. A method of inhibiting blood coagulation
comprising administering a protein of claim 92 with a
pharmaceutically acceptable carrier.

20 128. A method of inhibiting blood coagulation
comprising administering an AcaNAPc2 protein [SEQ. ID. NO.
59].

20 129. A protein of claim 73, wherein said protein has
two NAP domains.

25 130. A protein of claim 88, wherein said protein has
two NAP domains.

30 131. A protein of claim 92, wherein said protein has
two NAP domains.

30 132. An isolated protein having anticoagulant
activity, wherein said protein specifically inhibits the
catalytic activity of the fVIIa/TF complex in the presence
of fXa or catalytically inactive fXa derivative, and does
not specifically inhibit the activity of FVIIa in the
35 absence of TF and does not specifically inhibit
prothrombinase.

40 133. A protein of claim 132, wherein the protein is
AcaNAPc2 [SEQ. ID. NO. 59].

40 134. An isolated recombinant cDNA molecule encoding a
protein having anticoagulant activity, wherein said

5 protein specifically inhibits the catalytic activity of
the fVIIa/TF complex in the presence of fXa or
catalytically inactive fXa derivative, and does not
specifically inhibit the activity of FVIIa in the absence
of TF and does not specifically inhibit prothrombinase.

10

135. The cDNA molecule of claim 134, wherein the cDNA
codes for AcaNAPc2 [SEQ. ID. NO. 59].

136. An isolated cDNA molecule having a nucleotide
15 sequence substantially the same as AcaNAPc2 [SEQ. ID. NO.
19].

137. A protein having an amino acid sequence
substantially the same as AcaNAPc2 [SEQ. ID. NO. 59].

20

138. A protein of claim 1 wherein said NAP domain
includes the amino acid sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10

25 wherein

- (a) Cys-A1 is selected from SEQ. ID NOS. 83 and 205;
- (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS.
206 to 208;
- (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS.
30 209 to 222.
- (d) Cys-A5 is selected from SEQ. ID. NOS. 223 and
224;
- (e) Cys-A6 is selected from one of SEQ. ID. NOS. 225
to 227;
- 35 (f) Cys-A7-Cys-A8 is selected from one of SEQ. ID.
NOS. 228 to 229;
- (g) Cys-A9 is selected from one of SEQ. ID. NOS. 230
to 232; and
- (h) Cys-A10 is selected from one of SEQ. ID. NOS.
40 233 to 253.

- 5 139. An isolated protein having serine protease
inhibitory activity and having one or more NAP domains,
wherein each NAP domain includes the sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 [FORMULA IV],
10 wherein
 (a) A1 is an amino acid sequence of 7 to 8 amino
acid residues;
 (b) A2 is an amino acid sequence;
 (c) A3 is an amino acid sequence of 3 amino acid
15 residues;
 (d) A4 is an amino acid sequence;
 (e) A5 is an amino acid sequence of 3 to 4 amino
acid residues;
 (f) A6 is an amino acid sequence;
20 (g) A7 is an amino acid residue;
 (h) A8 is an amino acid sequence of 10 to 12 amino
acid residues; and
 (i) A9 is an amino acid sequence of 5 to 7 amino
acid residues;
25 (j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently
selected number of independently selected amino acid
residues and each sequence is selected such that each NAP
domain has in total less than about 120 amino acid
30 residues.
140. The protein of claim 139, wherein A3 has the
sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are
independently selected amino acid residues.
35
141. The protein of claim 139, wherein A3 is Glu-Pro-
Lys.
142. The protein of claim 139, wherein A4 is an amino
40 acid sequence having a net anionic charge.

5 143. The protein of claim 139, wherein A5 has the
sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are
independently selected amino acid residues.

 144. The protein of claim 143, wherein A5_a is Thr and
10 A5_c is Asn.

 145. The protein of claim 144, wherein A5 is selected
from Thr-Leu-Asn and Thr-Met-Asn.

15 146. The protein of claim 139, wherein A7 is Gln.

 147. The protein of claim 139 derived from a nematode
species.

20 148. The protein of claim 147, wherein said nematode
species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
polygyrus.

25 149. The protein of claim 139, wherein
 (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and
A3_b are independently selected amino acid residues;
 (b) A4 is an amino acid sequence having a net
30 anionic charge;
 (c) A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a
through A5_c are independently selected amino acid
residues; and
 (d) A7 is Gln.

35 150. The protein of claim 149 having a NAP domain
with an amino acid sequence that is substantially the same
as NAP domains selected from HpoNAP5 [SEQ. ID. NO. 60] and
NamNAP [SEQ. ID. NO. 61].

40 151. The protein of claim 149 derived from a nematode
species.

5

152. The protein of claim 151, wherein said nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

153. The protein of claim 139, wherein
(a) A3 is Glu-Pro-Lys;
(b) A4 is an amino acid sequence having a net
15 anionic charge;
(c) A5 is selected from Thr-Leu-Asn and Thr-Met-Asn;
and
(d) A7 is Gln.

20 154. The protein of claim 153 having a NAP domain with an amino acid sequence that is substantially the same as NAP domains selected from HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

25 155. The protein of claim 153 derived from a nematode species.

156. The protein of claim 155, wherein said nematode species is selected from the group consisting of
30 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

157. An isolated protein having serine protease
35 inhibitory activity and a NAP domain with an amino acid sequence substantially the same as NAP domains selected from the group consisting of HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

40 158. An isolated recombinant cDNA molecule encoding a protein having serine protease inhibitory activity and having one or more NAP domains, wherein each NAP domain

5 includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 [FORMULA IV],

wherein

- 10 (a) A1 is an amino acid sequence of 7 to 8 amino
acid residues;
- (b) A2 is an amino acid sequence;
- (c) A3 is an amino acid sequence of 3 amino acid
residues;
- (d) A4 is an amino acid sequence;
- 15 (e) A5 is an amino acid sequence of 3 to 4 amino
acid residues;
- (f) A6 is an amino acid sequence;
- (g) A7 is an amino acid residue;
- (h) A8 is an amino acid sequence of 10 to 12 amino
20 acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino
acid residues; and
- (j) A10 is an amino acid sequence;
- wherein each of A2, A4, A6 and A10 has an independently
25 selected number of independently selected amino acid
residues and each sequence is selected such that each NAP
domain has in total less than about 120 amino acid
residues.

30 159. The cDNA molecule of claim 158, wherein A3 is an
amino acid sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are
independently selected amino acid residues.

160. The cDNA molecule of claim 158, wherein A3 is
35 Glu-Pro-Lys.

161. The cDNA molecule of claim 158, wherein A4 is an
amino acid sequence having a net anionic charge.

40 162. The cDNA molecule of claim 158, wherein A5 has
the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are
independently selected amino acid residues.

5

163. The cDNA molecule of claim 162, wherein A5_a is Thr and A5_c is Asn.

164. The cDNA molecule of claim 163, wherein A5 is
10 selected from Thr-Leu-Asn and Thr-Met-Asn.

165. The cDNA molecule of claim 158, wherein A7 is Gln.

15 166. The cDNA molecule of claim 158 derived from a nematode species.

167. The cDNA molecule of claim 166, wherein said nematode species is selected from the group consisting of
20 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

168. The cDNA molecule of claim 158, wherein
25 (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
(b) A4 is an amino acid sequence having a net anionic charge;
(c) A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a
30 through A5_c are independently selected amino acid residues; and
(d) A7 is Gln.

169. The cDNA molecule of claim 168 having a
35 nucleotide sequence substantially the same as sequences selected from cDNAs coding for HpoNAP5 [SEQ. ID. NO. 14] and NamNAP [SEQ. ID. NO. 39].

170. The cDNA molecule of claim 168 derived from a
40 nematode species.

5 171. The cDNA molecule of claim 170, wherein said
nematode species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
polygyrus.

10

172. The cDNA molecule of claim 158, wherein
(a) A3 is Glu-Pro-Lys;
(b) A4 is an amino acid sequence having a net
anionic charge;

15 (c) A5 is selected from Thr-Leu-Asn and Thr-Met-Asn;
and

(d) A7 is Gln.

173. The cDNA molecule of claim 172 selected from
20 cDNAs coding for a protein having a NAP domain with an
amino acid sequence substantially the same as NAPs of
HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

174. The cDNA molecule of claim 172 derived from a
25 nematode species.

175. The cDNA molecule of claim 174, wherein said
nematode species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
30 *duodenale*, *Necator americanus*, and *Heligomosomoides*
polygyrus.

176. A cDNA molecule encoding a protein having serine
protease inhibitory activity selected from the group
35 consisting proteins having NAP domains substantially the
same as of HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID.
NO. 61].

177. A pharmaceutical composition comprising the
40 protein of claim 139.

178. A pharmaceutical composition comprising the

5 protein of claim 149.

179. A pharmaceutical composition comprising the protein of claim 153.

10 180. A pharmaceutical composition comprising a protein selected from the group consisting of HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

181. A method of inhibiting blood coagulation
15 comprising administering a protein of claim 139 with a pharmaceutically acceptable carrier.

182. A method of inhibiting blood coagulation comprising administering a protein of claim 149 with a
20 pharmaceutically acceptable carrier.

183. A method of inhibiting blood coagulation comprising administering a protein of claim 153 with a pharmaceutically acceptable carrier.

25 184. A method of inhibiting blood coagulation comprising administering a protein selected from the group consisting of HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

30 185. A protein of claim 139, wherein said protein has two NAP domains.

186. A protein of claim 149, wherein said protein has
35 two NAP domains.

187. A protein of claim 153, wherein said protein has two NAP domains.

40 188. A protein of claim 139 wherein said NAP domain includes the amino acid sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-

- 5 Cys-A9-Cys-A10
wherein
- (a) Cys-A1 is selected from SEQ. ID NOS. 86 and 254;
 - (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 255 to 257;
 - 10 (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 258 to 271.
 - (d) Cys-A5 is selected from SEQ. ID. NOS. 272 and 273;
 - (e) Cys-A6 is selected from one of SEQ. ID. NOS. 274
 - 15 to 276;
 - (f) Cys-A7-Cys-A8 is selected from one of SEQ. ID. NOS. 277 to 279;
 - (g) Cys-A9 is selected from one of SEQ. ID. NOS. 280 to 282; and
 - 20 (h) Cys-A10 is selected from one of SEQ. ID. NOS. 283 to 307.

189. An isolated protein having anticoagulant activity and having one or more NAP domains, wherein each

25 NAP domain includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 [FORMULA V],

wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino
- 30 acid residues;
- (b) A2 is an amino acid sequence;
- (c) A3 is an amino acid sequence of 3 amino acid residues;
- (d) A4 is an amino acid sequence;
- 35 (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
- (f) A6 is an amino acid sequence;
- (g) A7 is an amino acid residue;
- (h) A8 is an amino acid sequence of 11 to 12 amino
- 40 acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and

5 (j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently
selected number of independently selected amino acid
residues and each sequence is selected such that each NAP
domain has in total less than about 120 amino acid
10 residues.

190. The protein of claim 189, wherein A3 has the
sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are
independently selected amino acid residues.

15

191. The protein of claim 189, wherein A3 has the
sequence Glu-A3_a-A3_b, wherein A3_a is selected from the
group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and
Thr, and A3_b is selected from the group consisting of Lys,
20 Thr, and Arg.

192. The protein of claim 191, wherein A3 is selected
from the group consisting of
Glu-Ala-Lys,
25 Glu-Arg-Lys,
Glu-Pro-Lys,
Glu-Lys-Lys,
Glu-Ile-Thr,
Glu-His-Arg,
30 Glu-Leu-Lys, and
Glu-Thr-Lys.

193. The protein of claim 189, wherein A4 is an amino
acid sequence having a net anionic charge.

35

194. The protein of claim 189, wherein A7 is Val.

195. The protein of claim 189, wherein A7 is Ile.

40 196. The protein of claim 189, wherein A8 includes
the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ.
ID. NO. 68], wherein

- 5 (a) A8_a is the first amino acid residue in A8,
 (b) at least one of A8_a and A8_b is selected from the
 group consisting of Glu or Asp, and
 (c) A8_c through A8_g are independently selected amino
 acid residues.

10

197. The protein of claim 196, wherein

- (a) A8_a is Glu or Asp,
 (b) A8_b is an independently selected amino acid
 residue,
15 (c) A8_c is Gly,
 (d) A8_d is selected from the group consisting of
 Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
 (f) A8_f is Arg, and
20 (g) A8_g is selected from Asp and Asn.

198. The protein of claim 197, wherein A8_c-A8_d-A8_e-
A8_f-A8_g is selected from the group consisting of

- Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
25 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

30 199. The protein of claim 196, wherein

- (a) A8_a is an independently selected amino acid
 residue,
 (b) A8_b is Glu or Asp,
 (c) A8_c is Gly,
35 (d) A8_d is selected from the group consisting of
 Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
 (f) A8_f is Arg, and
 (g) A8_g is selected from Asp and Asn.

40

200. The protein of claim 199, wherein A8_c-A8_d-A8_e-
A8_f-A8_g is selected from the group consisting of

5 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
10 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

201. The protein of claim 196, wherein A8_c-A8_d-A8_e-
A8_f-A8_g is selected from the group consisting of

Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
15 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

202. The protein of claim 189, wherein A10 is
20 includes an amino acid sequence selected from the group
consisting of

Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
25 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

203. The protein of claim 202, wherein A10 includes
the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO.
74].

30

204. The protein of claim 203 having a NAP domain
with an amino acid sequence substantially the same as that
of AcaNAP5 [SEQ. ID. NO. 40] or AcaNAP6 [SEQ. ID. NO. 41].

35 205. The protein of claim 202, wherein A10 includes
the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO.
75].

206. The protein of claim 205 having a NAP domain
40 with an amino acid sequence substantially the same as that
of AcaNAP48 [SEQ. ID. NO. 42].

5 207. The protein of claim 202, wherein A10 includes the sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76].

 208. The protein of claim 207 having a NAP domain with an amino acid sequence substantially the same as a
10 NAP domain selected from NAP domains of AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AceNAP4 [SEQ. ID. NOS. 48 or 49].

15 209. The protein of claim 202, wherein A10 includes the sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

 210. The protein of claim 209 having a NAP domain with an amino acid sequence substantially the same as a
20 NAP domain selected from NAP domains of AcaNAP45 [SEQ. ID. NOS. 50 or 53], AcaNAP47 [SEQ. ID. NOS. 51 or 54], AduNAP7 [SEQ. ID. NOS. 52 or 56], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58].

25 211. The protein of claim 189 derived from a nematode species.

 212. The protein of claim 211, wherein said nematode species is selected from the group consisting of
30 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

 213. The protein of claim 189, wherein
35 (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
 (b) A4 is an amino acid sequence having a net anionic charge;
 (c) A7 is selected from the group consisting of Val
40 and Ile;
 (d) A8 includes an amino acid sequence selected from the group consisting of

5 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
10 (e) A10 includes an amino sequence selected from the
group consisting of
Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
15 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

214. The protein of claim 213 having a NAP domain
substantially the same as a NAP domain selected from the
group consisting of AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6
20 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23
[SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25
[SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31
[SEQ. ID. NO. 47], AceNAP4 [SEQ. ID. NOS. 48 or 49],
AcaNAP45 [SEQ. ID. NOS. 50 or 53], AcaNAP47 [SEQ. ID. NOS.
25 51 or 54], AduNAP7 [SEQ. ID. NOS. 52 or 56], AduNAP4 [SEQ.
ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ.
ID. NO. 58].

215. The protein of claim 213 derived from a nematode
30 species.

216. The protein of claim 215, wherein said nematode
species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
35 *duodenale*, *Necator americanus*, and *Heligomosomoides*
polygyrus.

217. The protein of claim 189, wherein
(a) A3 is selected from the group consisting of
40 Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,

- 5 Glu-Lys-Lys,
 Glu-Ile-Thr,
 Glu-His-Arg,
 Glu-Leu-Lys, and
 Glu-Thr-Lys;
- 10 (b) A4 is an amino acid sequence having a net
 anionic charge;
 (c) A7 is Val or Ile;
 (d) A8 includes an amino acid sequence selected from
 the group consisting of
- 15 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78],
 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81],
 and
- 20 A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82],
 wherein at least one of A8_a and A8_b is Glu or Asp;
 (e) A9 is an amino acid sequence of five amino acid
 residues; and
 (f) A10 includes an amino acid sequence selected
- 25 from the group consisting of
 Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

30

218. The protein of claim 217 having a NAP domain
substantially the same as a NAP domain selected from the
group consisting of AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6
[SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23
35 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25
[SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31
[SEQ. ID. NO. 47], AceNAP4 [SEQ. ID. NO. 48 or 49],
AcaNAP45 [SEQ. ID. NO. 50 or 53], AcaNAP47 [SEQ. ID. NO.
51 or 54], AduNAP7 [SEQ. ID. NO. 52 or 56], AduNAP4 [SEQ.
40 ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ.
ID. NO. 58].

5 219. The protein of claim 217 derived from a nematode species.

 220. The protein of claim 219, wherein said nematode species is selected from the group consisting of
10 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*.

 221. An isolated protein having anticoagulant
15 activity selected from the group consisting of AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AceNAP4
20 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], AduNAP7 [SEQ. ID. NO. 65], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58].

25 222. An isolated recombinant cDNA molecule encoding a protein having anticoagulant activity and having one or more NAP domains, wherein each NAP domain includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
30 Cys-A9-Cys-A10 [FORMULA V],

 wherein

 (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;

 (b) A2 is an amino acid sequence;

35 (c) A3 is an amino acid sequence of 3 amino acid residues;

 (d) A4 is an amino acid sequence;

 (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;

40 (f) A6 is an amino acid sequence;

 (g) A7 is an amino acid residue;

- 5 (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
- (j) A10 is an amino acid sequence;
- 10 wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid residues.
- 15 223. The cDNA molecule of claim 222, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues.
- 20 224. The cDNA molecule of claim 222, wherein A3 is an amino acid sequence Glu-A3_a-A3_b, wherein A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg.
- 25 225. The cDNA molecule of claim 224, wherein A3 is selected from the group consisting of
- Glu-Ala-Lys,
- Glu-Arg-Lys,
- 30 Glu-Pro-Lys,
- Glu-Lys-Lys,
- Glu-Ile-Thr,
- Glu-His-Arg,
- Glu-Leu-Lys, and
- 35 Glu-Thr-Lys.
226. The cDNA molecule of claim 222, wherein A4 is an amino acid sequence having a net anionic charge.
- 40 227. The cDNA molecule of claim 222, wherein A7 is Val.

5 228. The cDNA molecule of claim 222, wherein A7 is Ile.

 229. The cDNA molecule of claim 222, wherein A8 includes an amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-
10 A8_g, [SEQ. ID. NO. 68] wherein
 (a) A8_a is the first amino acid residue in A8,
 (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
 (c) A8_c through A8_g are independently selected amino
15 acid residues.

 230. The cDNA molecule of claim 229, wherein
 (a) A8_a is Glu or Asp,
 (b) A8_b is an independently selected amino acid
20 residue,
 (c) A8_c is Gly,
 (d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,
 (e) A8_e is Tyr,
25 (f) A8_f is Arg, and
 (g) A8_g is selected from Asp and Asn.

 231. The cDNA molecule of claim 230, wherein A8_c-A8_d-A8_e-A8_f-A8_g is selected from the group consisting of
30 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

35 232. The cDNA molecule of claim 229, wherein
 (a) A8_a is an independently selected amino acid residue,
 (b) A8_b is Glu or Asp,
40 (c) A8_c is Gly,
 (d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,

- 5 (e) A8_e is Tyr,
 (f) A8_f is Arg, and
 (g) A8_g is selected from Asp and Asn.

233. The cDNA molecule of claim 232, wherein A8_c-A8_d-
10 A8_e-A8_f-A8_g is selected from the group consisting of
 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
15 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

234. The cDNA molecule of claim 229, wherein A8_c-A8_d-
A8_e-A8_f-A8_g is selected from the group consisting of
 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
20 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

25 235. The cDNA molecule of claim 222, wherein A10
includes an amino acid sequence selected from the group
consisting of
 Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
30 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

236. The cDNA molecule of claim 235, wherein A10
includes the sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO.
35 74].

237. The cDNA molecule of claim 236, having a
nucleotide sequence substantially the same as that coding
for AcaNAP5 [SEQ. ID. NO. 3] or AcaNAP6 [SEQ. ID. NO. 5].
40

5 238. The cDNA molecule of claim 235, wherein A10
includes the sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO.
75].

 239. The cDNA molecule of claim 238, having a
10 nucleotide sequence substantially the same as that coding
for AcaNAP48 [SEQ. ID. NO. 38].

 240. The cDNA molecule of claim 235, wherein A10
includes the sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID.
15 NO. 76].

 241. The cDNA molecule of claim 240 having a
nucleotide sequence substantially the same as that
selected from the group consisting of cDNAs coding for
20 AcaNAP23 [SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32],
AcaNAP25 [SEQ. ID. NO. 33], AcaNAP44 [SEQ. ID. NO. 35],
AcaNAP31 [SEQ. ID. NO. 34], and AceNAP4 [SEQ. ID. NO. 9].

 242. The cDNA molecule of claim 235, wherein A10
25 includes the sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO.
77].

 243. The cDNA molecule of claim 242 having a
nucleotide sequence substantially the same as that
30 selected from the group consisting of cDNAs coding for
AcaNAP45 [SEQ. ID. NO. 36], AcaNAP47 [SEQ. ID. NO. 37],
AduNAP7 [SEQ. ID. NO. 13], AduNAP4 [SEQ. ID. NO. 12],
AceNAP5 [SEQ. ID. NO. 10], and AceNAP7 [SEQ. ID. NO. 11].

35 244. The cDNA molecule of claim 222 derived from a
nematode species.

 245. The cDNA molecule of claim 244, wherein said
nematode species is selected from the group consisting of
40 *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
polygyrus.

5

246. The cDNA molecule of claim 222, wherein

(a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;

(b) A4 is an amino acid sequence having a net
10 anionic charge;

(c) A7 is selected from the group consisting of Val and Ile;

(d) A8 includes an amino acid sequence selected from the group consisting of

15 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and

20 (e) A10 includes an amino sequence selected from the group consisting of

Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
25 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

247. The cDNA molecule of claim 246 having a nucleotide sequence substantially the same as that selected from the group consisting of cDNAs coding for

30 AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5],
AcaNAP48 [SEQ. ID. NO. 38], AcaNAP23 [SEQ. ID. NO. 31],
AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33],
AcaNAP44 [SEQ. ID. NO. 35], AcaNAP31 [SEQ. ID. NO. 34],
AceNAP4 [SEQ. ID. NO. 9], AcaNAP45 [SEQ. ID. NO. 36],
35 AcaNAP47 [SEQ. ID. NO. 37], AduNAP7 [SEQ. ID. NO. 13],
AduNAP4 [SEQ. ID. NO. 12], AceNAP5 [SEQ. ID. NO. 10], and
AceNAP7 [SEQ. ID. NO. 11].

248. The cDNA molecule of claim 246 derived from a
40 nematode species.

5 249. The cDNA molecule of claim 248, wherein said
nematode species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
polygyrus.

10

250. The cDNA molecule of claim 222, wherein

(a) A3 is selected from the group consisting of

Glu-Ala-Lys,

Glu-Arg-Lys,

15

Glu-Pro-Lys,

Glu-Lys-Lys,

Glu-Ile-Thr,

Glu-His-Arg,

Glu-Leu-Lys, and

20

Glu-Thr-Lys;

(b) A4 is an amino acid sequence having a net
anionic charge;

(c) A7 is Val or Ile;

(d) A8 is selected from the group consisting of

25

A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78],

A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79],

A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80],

A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81],

and

30

A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82],

wherein at least one of A8_a and A8_b is Glu or Asp;

(e) A9 is an amino acid sequence of five amino acid
residues; and

(f) A10 includes an amino acid sequence selected

35

from the group consisting of

Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],

Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],

Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and

Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

40

251. The cDNA molecule of claim 250 that is selected
from the group consisting of cDNAs coding for AcaNAP5

5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5], AcaNAP48 [SEQ.
ID. NO. 38], AcaNAP23 [SEQ. ID. NO. 31], AcaNAP24 [SEQ.
ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33], AcaNAP44 [SEQ.
ID. NO. 35], AcaNAP31 [SEQ. ID. NO. 34], AceNAP4 [SEQ. ID.
NO. 9], AcaNAP45 [SEQ. ID. NO. 36], AcaNAP47 [SEQ. ID. NO.
10 37], AduNAP7 [SEQ. ID. NO. 13], AduNAP4 [SEQ. ID. NO. 12],
AceNAP5 [SEQ. ID. NO. 10], and AceNAP7 [SEQ. ID. NO. 11].

252. The cDNA molecule of claim 250 derived from a
nematode species.

15

253. The cDNA molecule of claim 252, wherein said
nematode species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
20 *polygyrus*.

254. A cDNA molecule encoding a protein having
anticoagulant activity selected from the group consisting
of cDNAs substantially the same as cDNAs coding for

25 AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5],
AcaNAP48 [SEQ. ID. NO. 38], AcaNAP23 [SEQ. ID. NO. 31],
AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33],
AcaNAP44 [SEQ. ID. NO. 35], AcaNAP31 [SEQ. ID. NO. 34],
AceNAP4 [SEQ. ID. NO. 9], AcaNAP45 [SEQ. ID. NO. 36],
30 AcaNAP47 [SEQ. ID. NO. 37], AduNAP7 [SEQ. ID. NO. 13],
AduNAP4 [SEQ. ID. NO. 12], AceNAP5 [SEQ. ID. NO. 10], and
AceNAP7 [SEQ. ID. NO. 11].

255. A pharmaceutical composition comprising a
35 protein of claim 189.

256. A pharmaceutical composition comprising a
protein of claim 213.

40 257. A pharmaceutical composition comprising a
protein of claim 217.

5 258. A pharmaceutical composition comprising a
protein having a NAP domain substantially the same as a
NAP domain selected from the group consisting of AcaNAP5
[SEQ. ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48
[SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24
10 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44
[SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AceNAP4
[SEQ. ID. NOS. 48 or 49], AcaNAP45 [SEQ. ID. NOS. 50 or
53], AcaNAP47 [SEQ. ID. NOS. 51 or 54], AduNAP7 [SEQ. ID.
NO. 52 or 56], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ.
15 ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58].

 259. A method of inhibiting blood coagulation
comprising administering a protein of claim 189 with a
pharmaceutically acceptable carrier.

20

 260. A method of inhibiting blood coagulation
comprising administering a protein of claim 213 with a
pharmaceutically acceptable carrier.

25 261. A method of inhibiting blood coagulation
comprising administering a protein of claim 217 with a
pharmaceutically acceptable carrier.

 262. A method of inhibiting blood coagulation
30 comprising administering a protein having a NAP domain
substantially the same as NAP domains selected from the
group consisting of AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6
[SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23
[SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25
35 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31
[SEQ. ID. NO. 47], AceNAP4 [SEQ. ID. NOS. 48 and 49],
AcaNAP45 [SEQ. ID. NOS. 50 and 53], AcaNAP47 [SEQ. ID.
NOS. 51 and 54], AduNAP7 [SEQ. ID. NOS. 52 and 56],
AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and
40 AceNAP7 [SEQ. ID. NO. 58].

 263. A protein of claim 189, wherein said protein has

5 two NAP domains.

264. A protein of claim 213, wherein said protein has two NAP domains.

10 265. A protein of claim 217, wherein said protein has two NAP domains.

266. A protein having two NAP domains, wherein said protein is selected from the group consisting of AceNAP4
15 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65].

267. A protein of claim 1 wherein said NAP domain includes the amino acid sequence:
20 Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10
wherein
(a) Cys-A1 is selected from SEQ. ID NOS. 87 and 308;
(b) Cys-A2-Cys is selected from one of SEQ. ID. NOS.
25 309 to 311;
(c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 312 to 325.
(d) Cys-A5 is selected from SEQ. ID. NOS. 326 and 327;
30 (e) Cys-A6 is selected from one of SEQ. ID. NOS. 328 to 330;
(f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 331 and 332;
(g) Cys-A9 is selected from one of SEQ. ID. NOS. 333
35 to 335; and
(h) Cys-A10 is selected from one of SEQ. ID. NOS. 336 to 356.

268. An oligonucleotide comprising a nucleotide
40 sequence selected from

YG109: TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.

5 88], and

YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]

269. An oligonucleotide comprising a nucleotide
sequence selected from

10

NAP-1: AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ. ID. NO.
90] and

NAP-4.RC TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ. ID.
15 NO. 91].

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Figur 1

```

      1           10           20           30
      *           *           *           *
G AATTC CGCTA CTACTCAACA ATG AAG ATG CTT TAC GCT ATC GCT
      Met Lys Met Leu Tyr Ala Ile Ala

      40           50           60           70
      *           *           *           *
ATA ATG TTT CTC CTG GTA TCA TTA TGC AGC GCA AGA ACA GTG
Ile Met Phe Leu Leu Val Ser Leu Cys Ser Ala Arg Thr Val

      80           90           100          110          120
      *           *           *           *           *
AGG AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC
Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp

      130          140          150          160
      *           *           *           *
GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG
Asp Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys Cys Asn Glu

      170          180          190          200
      *           *           *           *
GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC TCA CGT GGT
Glu Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg Ser Arg Gly

      210          220          230          240
      *           *           *           *
TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC TAC
Cys Leu Leu Pro Pro Ala Cys Val Cys Lys Asp Gly Phe Tyr

      250          260          270          280
      *           *           *           *
AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC
Arg Asp Thr Val Ile Gly Asp Cys Val Arg Glu Glu Glu Cys

      290          300          310          320          330
      *           *           *           *           *
GAC CAA CAT GAG ATT ATA CAT GTC TGA ACGAGAAAGC AACAATAACC
Asp Gln His Glu Ile Ile His Val

      340          350          360          370          380
      *           *           *           *           *
AAAGGTTCCA ACTCTCGCTC TGCAAAATCG CTAGTTGGAT GTCTCTTTTG

      390          400          410          420          430
      *           *           *           *           *
CGTCCGAATA GTTTTAGTTG ATGTTAAGTA AGAACTCCTG CTGGAGAGAA

      440          450
      *           *
TAAAGCTTTC CAACTCC poly(A)

```

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Figure 2

Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Asp
 1 5 10

Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys Cys Asn Glu Glu
 15 20 25

Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg Ser Arg Gly Cys
 30 35 40

Leu Leu Pro Pro Ala Cys Val Cys Lys Asp Gly Phe Tyr Arg
 45 50 55

Asp Thr Val Ile Gly Asp Cys Val Arg Glu Glu Glu Cys Asp
 60 65 70

Gln His Glu Ile Ile His Val
 75

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Figure 3

```

      1      10      20      30
      *      *      *      *
G AATTCGCTA CTACTCAACA ATG AAG ATG CTT TAC GCT ATC GCT
                               Met Lys Met Leu Tyr Ala Ile Ala

      40      50      60      70
      *      *      *      *
ATA ATG TTT CTC CTG GTG TCA TTA TGC AGC ACA AGA ACA GTG
Ile Met Phe Leu Leu Val Ser Leu Cys Ser Thr Arg Thr Val

      80      90      100      110      120
      *      *      *      *      *
AGG AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC
Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp

      130      140      150      160
      *      *      *      *
GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC AAG TGC AGT GAG
Val Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys Cys Ser Glu

      170      180      190      200
      *      *      *      *
GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG
Glu Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe Ser Cys Pro

      210      220      230      240
      *      *      *      *
GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA TTC TAC AGA GAC
Gly Pro Ala Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg Asp

      250      260      270      280
      *      *      *      *
ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC CAA
Thr Val Ile Gly Asp Cys Val Lys Glu Glu Glu Cys Asp Gln

      290      300      310      320      330
      *      *      *      *      *
CAT GAG ATT ATT CAT GTC TGA ACGAGAGAGC AGTAATAACC
His Glu Ile Ile His Val

      340      350      360      370      380
      *      *      *      *      *
AAAGGTTCCA ACTTTCGCTC TACAAAATCG CTAGTTGGAT TTCTCCTTTG

      390      400      410      420      430
      *      *      *      *      *
CGTGCGAATA GTTTTAGTTG ATATTAAGTA AAACCTCCTG TTGAAGAGAA

      440
      *
TAAAGCTTTC CAACTTC poly(A)

```

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Figure 4

Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Val
 1 5 10

Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys Cys Ser Glu Glu
 15 20 25

Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe Ser Cys Pro Gly
 30 35 40

Pro Ala Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg Asp Thr
 45 50 55

Val Ile Gly Asp Cys Val Lys Glu Glu Glu Cys Asp Gln His
 60 65 70

Glu Ile Ile His Val
 75

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Figure 5

Arg Thr Val Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu
1 5 10

Trp Leu Asp Asp Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys
15 20

Cys Asn Glu Glu Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg
25 30 35

Ser Arg Gly Cys Leu Leu Pro Pro Ala Cys Val Cys Lys Asp
40 45 50

Gly Phe Tyr Arg Asp Thr Val Ile Gly Asp Cys Val Arg Glu
55 60 65

Glu Glu Cys Asp Gln His Glu Ile Ile His Val
70 75

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Figure 6

Arg Thr Val Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu
1 5 10

Trp Leu Asp Val Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys
15 20

Cys Ser Glu Glu Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe
25 30 35

Ser Cys Pro Gly Pro Ala Ala Cys Val Cys Glu Asp Gly Phe
40 45 50

Tyr Arg Asp Thr Val Ile Gly Asp Cys Val Lys Glu Glu Glu
55 60 65

Cys Asp Gln His Glu Ile Ile His Val
70 75

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Figure 7A-1

```

      1      10      20      30      40
      *      *      *      *      *
  GAATTCACTA TTATCCAACA ATG GCG GTG CTT TAT TCA GTA GCA
  EcoRI                      Met Ala Val Leu Tyr Ser Val Ala

      50      60      70      80
      *      *      *      *
  ATA GCG TTA CTA CTG GTA TCA CAA TGC AGT GGG AAA CCG AAC
  Ile Ala Leu Leu Leu Val Ser Gln Cys Ser Gly Lys Pro Asn

      90      100      110      120
      *      *      *      *
  AAT GTG ATG ACT AAC GCT TGT GGT CTT AAT GAA TAT TTC GCT
  Asn Val Met Thr Asn Ala Cys Gly Leu Asn Glu Tyr Phe Ala

      130      140      150      160      170
      *      *      *      *      *
  GAG TGT GGC AAT ATG AAG GAA TGC GAG CAC AGA TGC AAT GAG
  Glu Cys Gly Asn Met Lys Glu Cys Glu His Arg Cys Asn Glu

      180      190      200      210
      *      *      *      *
  GAG GAA AAT GAG GAA AGG GAC GAG GAA AGA ATA ACG GCA TGC
  Glu Glu Asn Glu Glu Arg Asp Glu Glu Arg Ile Thr Ala Cys

      220      230      240      250
      *      *      *      *
  CTC ATC CGT GTG TGT TTC CGT CCT GGT GCT TGC GTA TGC AAA
  Leu Ile Arg Val Cys Phe Arg Pro Gly Ala Cys Val Cys Lys

      260      270      280      290
      *      *      *      *
  GAC GGA TTC TAT AGA AAC AGA ACA GGC AGC TGT GTG GAA GAA
  Asp Gly Phe Tyr Arg Asn Arg Thr Gly Ser Cys Val Glu Glu

      300      310      320      330
      *      *      *      *
  GAT GAC TGC GAG TAC GAG AAT ATG GAG TTC ATT ACT TTT GCA
  Asp Asp Cys Glu Tyr Glu Asn Met Glu Phe Ile Thr Phe Ala

      340      350      360      370      380
      *      *      *      *      *
  CCA GAA GTA CCG ATA TGT GGT TCC AAC GAA AGG TAC TCC GAC
  Pro Glu Val Pro Ile Cys Gly Ser Asn Glu Arg Tyr Ser Asp

      390      400      410      420
      *      *      *      *
  TGC GGC AAT GAC AAA CAA TGC GAG CGC AAA TGC AAC GAG GAC
  Cys Gly Asn Asp Lys Gln Cys Glu Arg Lys Cys Asn Glu Asp

      430      440      450      460
      *      *      *      *
  GAT TAT GAG AAG GGA GAT GAG GCA TGC CGC TCA CAT GTT TGT
  Asp Tyr Glu Lys Gly Asp Glu Ala Cys Arg Ser His Val Cys

```

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Figur 7A-2

```

      470      480      490      500
      *      *      *      *
GAA CGT CCT GGT GCC TGT GTA TGC GAA GAC GGG TTC TAC AGA
Glu Arg Pro Gly Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg

      510      520      530      540
      *      *      *      *
AAC AAA AAA GGT AGC TGT GTG GAA AGC GAT GAC TGC GAA TAC
Asn Lys Lys Gly Ser Cys Val Glu Ser Asp Asp Cys Glu Tyr

      550      560      570      580      590
      *      *      *      *      *
GAT AAT ATG GAT TTC ATC ACT TTT GCA CCA GAA ACC TCA CGA
Asp Asn Met Asp Phe Ile Thr Phe Ala Pro Glu Thr Ser Arg

      600      610      620      630      640
      *      *      *      *      *
TAA CCAAAGATGC TACCTCTCGT ACGCAACTCC GCTGATTGAGGTTGATTC

      650      660      670      680      690
      *      *      *      *      *
ACTCCCTTGCATCTCAACATTTTTTTTGTGATGCTGTGCATCTGAGCTTAACCTG

      700      710
      *      *
ATAAAGCCTATGGTG poly(A)

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Figure 7B

```

      1          10          20          30          40
      *          *          *          *          *
  GAATTC CGC ATG CGG ACG CTC TAC CTC ATT TCT ATC TGG TTG
  EcoRI      Met Arg Thr Leu Tyr Leu Ile Ser Ile Trp Leu

          50          60          70          80
          *          *          *          *
  TTC CTC ATC TCG CAA TGT AAT GGA AAA GCA TTC CCG AAA TGT
  Phe Leu Ile Ser Gln Cys Asn Gly Lys Ala Phe Pro Lys Cys

          90          100          110          120
          *          *          *          *
  GAC GTC AAT GAA AGA TTC GAG GTG TGT GGC AAT CTG AAG GAG
  Asp Val Asn Glu Arg Phe Glu Val Cys Gly Asn Leu Lys Glu

          130          140          150          160
          *          *          *          *
  TGC GAG CTC AAG TGC GAT GAG GAC CCT AAG ATA TGC TCT CGT
  Cys Glu Leu Lys Cys Asp Glu Asp Pro Lys Ile Cys Ser Arg

          170          180          190          200          210
          *          *          *          *          *
  GCA TGT ATT CGT CCC CCT GCT TGC GTA TGC GAT GAC GGA TTC
  Ala Cys Ile Arg Pro Pro Ala Cys Val Cys Asp Asp Gly Phe

          220          230          240          250
          *          *          *          *
  TAC AGA GAC AAA TAT GGC TTC TGT GTT GAA GAA GAC GAA TGT
  Tyr Arg Asp Lys Tyr Gly Phe Cys Val Glu Glu Asp Glu Cys

          260          270          280          290
          *          *          *          *
  AAC GAT ATG GAG ATT ATT ACT TTT CCA CCA GAA ACC AAA TGA
  Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu Thr Lys

          300          310          320          330          340
          *          *          *          *          *
  TGACCGAAGC TTCCACCTTT CTATACATAT CTTCACTGCTTGACAGGCTTCT

          350          360          370          380          390          400
          *          *          *          *          *          *
  CGACAATTTAGAAAGTTCTGCTTGACTTTGTCTATTTGAAATTGTTCACTAATG

          410          420
          *          *
  GGGGAAGTAAAGCATTTTCACGAC poly(A)

```

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Figure 7C

```

1      10      20      30      40
*      *      *      *      *
GAATTCGCT ACATTTTCAA CA ATG TCG ACG CTT TAT GTT ATC
EcoRI                      Met Ser Thr Leu Tyr Val Ile

      50      60      70      80
      *      *      *      *
GCA ATA TGT TTG CTG CTT GTT TCG CAA TGC AAT GGA AGA ACG
Ala Ile Cys Leu Leu Leu Val Ser Gln Cys Asn Gly Arg Thr

      90      100     110     120
      *      *      *      *
GTG AAG AAG TGT GGC AAG AAT GAA AGA TAC GAC GAC TGT GGC
Val Lys Lys Cys Gly Lys Asn Glu Arg Tyr Asp Asp Cys Gly

130      140      150      160
*      *      *      *
AAT GCA AAG GAC TGC GAG ACC AAG TGC GGT GAA GAG GAA AAG
Asn Ala Lys Asp Cys Glu Thr Lys Cys Gly Glu Glu Glu Lys

170      180      190      200      210
*      *      *      *      *
GTG TGC CGT TCG CGT GAG TGT ACT AGT CCT GGT GCC TGC GTA
Val Cys Arg Ser Arg Glu Cys Thr Ser Pro Gly Ala Cys Val

      220      230      240      250
      *      *      *      *
TGC GAA CAA GGA TTC TAC AGA GAT CCG GCT GGC GAC TGT GTC
Cys Glu Gln Gly Phe Tyr Arg Asp Pro Ala Gly Asp Cys Val

      260      270      280      290
      *      *      *      *
ACT GAT GAA GAA TGT GAT GAA TGG AAC AAT ATG GAG ATC ATT
Thr Asp Glu Glu Cys Asp Glu Trp Asn Asn Met Glu Ile Ile

      300      310      320      330      340
      *      *      *      *      *
ACT ATG CCA AAA CAG TAG TGCGAAGTTC CCTTCTTTCT CCAAATCTG
Thr Met Pro Lys Gln

      350      360      370      380      390
      *      *      *      *      *
C TCCGTGCTCAATTATCACACACCTCCACTAGTTAAGATTGACTGACTCTCTTG

      400      410      420      430      440      450
      *      *      *      *      *      *
CATTGTAGTATTTTCGCTTGACTCTGTGCATTTAAGCATGAGATACTACTAGGGA

      460      470
      *      *
GAATAAAAATTACTAACTAC poly(A)

```

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Figure 7D

```

1      10      20      30      40
*      *      *      *      *
GAATTCGG AAA TGT CCT ACC GAT GAA TGG TTC GAT TGG TGT
EcoRI      Lys Cys Pro Thr Asp Glu Trp Phe Asp Trp Cys

50      60      70      80
*      *      *      *
GGA ACT TAC AAG CAT TGC GAA CTC AAG TGC GAT AGG GAG CTA
Gly Thr Tyr Lys His Cys Glu Leu Lys Cys Asp Arg Glu Leu

90      100     110     120
*      *      *      *
ACT GAG AAA GAA GAG CAG GCA TGT CTC TCA CGT GTT TGT GAG
Thr Glu Lys Glu Glu Gln Ala Cys Leu Ser Arg Val Cys Glu

130     140     150     160
*      *      *      *
AAG TCC GCT TGC GTA TGC AAT GAC GGA TTA TAC AGA GAC AAG
Lys Ser Ala Cys Val Cys Asn Asp Gly Leu Tyr Arg Asp Lys

170     180     190     200     210
*      *      *      *      *
TTT GGC AAC TGT GTT GAA AAA GAC GAA TGC AAC GAT ATG GAG
Phe Gly Asn Cys Val Glu Lys Asp Glu Cys Asn Asp Met Glu

220     230     240     250
*      *      *      *
ATT ATT ACT TTT GCA CCA GAA ACC AAA TAA TGGCCTAAGG TTCC
Ile Ile Thr Phe Ala Pro Glu Thr Lys

260     270     280     290     300
*      *      *      *      *
AAACCT TGCTACACAC CGTCAGTGCTTTACTGTTTCCTCTACGTGTTAGTAGT

310     320     330     340     350     360
*      *      *      *      *
TTTGCTTGACTCTGTGTATTTAAGCATTGTCTACTAATGGGCAAAGTAAAGCATT

370     380     390
*      *      *
GTAAGGACATAATAATGAGTAAACCTTCTGATTT poly(A)

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Figure 7E-1

```

1      10      20      30      40
*      *      *      *      *
GAATTCGGG CGGCAGAAAG ATG CGA ATG CTC TAC CTT GTT CCT
EcoRI                      Met Arg Met Leu Tyr Leu Val Pro

50      60      70      80
*      *      *      *
ATC TGG TTG CTG CTC ATT TCG CTA TGC AGT GGA AAA GCT GCG
Ile Trp Leu Leu Leu Ile Ser Leu Cys Ser Gly Lys Ala Ala

90      100     110     120
*      *      *      *
AAG AAA TGT GGT CTC AAT GAA AGG CTG GAC TGT GGC AAT CTG
Lys Lys Cys Gly Leu Asn Glu Arg Leu Asp Cys Gly Asn Leu

130     140     150     160     170
*      *      *      *      *
AAG CAA TGC GAG CCC AAG TGC AGC GAC TTG GAA AGT GAG GAG
Lys Gln Cys Glu Pro Lys Cys Ser Asp Leu Glu Ser Glu Glu

180     190     200     210
*      *      *      *
TAT GAG GAG GAA GAT GAG TCG AAA TGT CGA TCA CGT GAA TGT
Tyr Glu Glu Glu Asp Glu Ser Lys Cys Arg Ser Arg Glu Cys

220     230     240     250
*      *      *      *
TCT CGT CGT GTT TGT GTA TGC GAT GAA GGA TTC TAC AGA AAC
Ser Arg Arg Val Cys Val Cys Asp Glu Gly Phe Tyr Arg Asn

260     270     280     290
*      *      *      *
AAG AAG GGC AAG TGT GTT GCA AAA GAT GTT TGC GAG GAC GAC
Lys Lys Gly Lys Cys Val Ala Lys Asp Val Cys Glu Asp Asp

300     310     320     330
*      *      *      *
AAT ATG GAG ATT ATC ACT TTT CCA CCA GAA GAC GAA TGT GGT
Asn Met Glu Ile Ile Thr Phe Pro Pro Glu Asp Glu Cys Gly

340     350     360     370     380
*      *      *      *      *
CCC GAT GAA TGG TTC GAC TAC TGT GGA AAT TAT AAG AAG TGC
Pro Asp Glu Trp Phe Asp Tyr Cys Gly Asn Tyr Lys Lys Cys

390     400     410     420
*      *      *      *
GAA CGC AAG TGC AGT GAG GAG ACA AGT GAG AAA AAT GAG GAG
Glu Arg Lys Cys Ser Glu Glu Thr Ser Glu Lys Asn Glu Glu

430     440     450     460
*      *      *      *
GCA TGC CTC TCT CGT GCT TGT ACT GGT CGT GCT TGC GTA TGC
Ala Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys

```


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Figure 7E-2

470 480 490 500
* * * *
AAA GAC GGA TTG TAC AGA GAC GAC TTT GGC AAC TGT GTT CCA
Lys Asp Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Pro

510 520 530 540
* * * *
CAT GAC GAA TGC AAC GAT ATG GAG ATC ATC ACT TTT CCA CCG
His Asp Glu Cys Asn Asp Met Glu Ile Ile Thr Phe Pro Pro

550 560 570 580 590
* * * * *
GAA ACC AAA CAT TGA CCAGAGGCTC CAACTCTCGC TACACAACGT CA
Glu Thr Lys His

600 610 620 630 640 650
* * * * * *
GGGCTAGAATGGCCCCTCTGCGAGTTAGTAGTTTGTGCTTGACTCTGCTTATTGTA

660 670 680
* * *
GCACTTTCTATTGATGGCGAAAATAAAGCATTATAAAC poly(A)

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Figure 7F

1 10 20 30 40
 * * * * *
GAATTCCGCG CACCTGAGAG GTGAGCTACG CAAGTCTTCG CTGGTACA
 EcoRI

50 60 70 80 90
 * * * * *
 ATG ATC CGA AAG CTC GTT CTG CTG ACT GCT ATC GTC ACG GTG
 Met Ile Arg Lys Leu Val Leu Leu Thr Ala Ile Val Thr Val

100 110 120 130
 * * * *
 GTG CTA AGT GCG AAG ACC TGT GGA CCA AAC GAG GAG TAC ACT
 Val Leu Ser Ala Lys Thr Cys Gly Pro Asn Glu Glu Tyr Thr

140 150 160 170
 * * * *
 GAA TGC GGG ACG CCA TGC GAG CCG AAG TGC AAT GAA CCG ATG
 Glu Cys Gly Thr Pro Cys Glu Pro Lys Cys Asn Glu Pro Met

180 190 200 210
 * * * *
 CCA GAC ATC TGT ACT CTG AAC TGC ATC GTG AAC GTG TGT CAG
 Pro Asp Ile Cys Thr Leu Asn Cys Ile Val Asn Val Cys Gln

220 230 240 250
 * * * *
 TGC AAA CCC GGC TTC AAG CGC GGA CCG AAA GGA TGC GTC GCC
 Cys Lys Pro Gly Phe Lys Arg Gly Pro Lys Gly Cys Val Ala

260 270 280 290 300
 * * * * *
 CCC GGA CCA GGC TGT AAA TAG TTCTCCACCT GCCCTTTCGT TGGAA
 Pro Gly Pro Gly Cys Lys

310 320 330 340
 * * * *
 CAAAT GGCTGTCTTTTTACATTCTGAATCAATAAAGCCGAACGGT poly(A)

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Figure 8A

```

1      10      20      30      40
*      *      *      *      *
AAGCTT TGCT AACATACTGC GTAATAAGGA GTCTTAATC ATG CCA GTT
HindIII                               Met Pro Val

50      60      70      80      90
*      *      *      *      *
CTT TTG GGT ATT CCG TTA TTA TTG CGT TTC CTC GGT TTC CTT
Leu Leu Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu

100     110     120     130
*      *      *      *
CTG GTA ACT TTG TTC GGC TAT CTG CTT ACT TTC CTT AAA AAG
Leu Val Thr Leu Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys

140     150     160     170
*      *      *      *
GGC TTC GGT AAG ATA GCT ATT GCT ATT TCA TTG TTT CTT GCT
Gly Phe Gly Lys Ile Ala Ile Ala Ile Ser Leu Phe Leu Ala

180     190     200     210
*      *      *      *
CTT ATT ATT GGG CTT AAC TCA ATT CTT GTG GGT TAT CTC TCT
Leu Ile Ile Gly Leu Asn Ser Ile Leu Val Gly Tyr Leu Ser

220     230     240     250
*      *      *      *
GAT ATT AGC GCA CAA TTA CCC TCT GAT TTT GTT CAG GGC GTT
Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe Val Gln Gly Val

260     270     280     290     300
*      *      *      *      *
CAG TTA ATT CTC CCG TCT AAT GCG CTT CCC TGT TTT TAT GTT
Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe Tyr Val

310     320     330     340
*      *      *      *
ATT CTC TCT GTA AAG GCT GCT ATT TTC ATT TTT GAC GTT AAA
Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys

350     360     370     380
*      *      *      *
CAA AAA ATC GTT TCT TAT TTG GAT TGG GAT AAA GGT GGA GGC
Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly

390     400     410     420     430
*      *      *      *      *
TCA GGC GGA GGCCAAGTCGGCC ATCCCATATCAC GCGGCCGC GGATCC
Ser Gly Gly      SfiI                      NotI      BamHI

```

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Figure 8B

```

1      10      20      30      40
*      *      *      *      *
AAGCTT TGCT AACATACTGC GTAATAAGGA GTCTTAATC ATG CCA GTT
HindIII                               Met Pro Val

50      60      70      80      90
*      *      *      *      *
CTT TTG GGT ATT CCG TTA TTA TTG CGT TTC CTC GGT TTC CTT
Leu Leu Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu

100      110      120      130
*      *      *      *
CTG GTA ACT TTG TTC GGC TAT CTG CTT ACT TTC CTT AAA AAG
Leu Val Thr Leu Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys

140      150      160      170
*      *      *      *
GGC TTC GGT AAG ATA GCT ATT GCT ATT TCA TTG TTT CTT GCT
Gly Phe Gly Lys Ile Ala Ile Ala Ile Ser Leu Phe Leu Ala

180      190      200      210
*      *      *      *
CTT ATT ATT GGG CTT AAC TCA ATT CTT GTG GGT TAT CTC TCT
Leu Ile Ile Gly Leu Asn Ser Ile Leu Val Gly Tyr Leu Ser

220      230      240      250
*      *      *      *
GAT ATT AGC GCA CAA TTA CCC TCT GAT TTT GTT CAG GGC GTT
Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe Val Gln Gly Val

260      270      280      290      300
*      *      *      *      *
CAG TTA ATT CTC CCG TCT AAT GCG CTT CCC TGT TTT TAT GTT
Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe Tyr Val

310      320      330      340
*      *      *      *
ATT CTC TCT GTA AAG GCT GCT ATT TTC ATT TTT GAC GTT AAA
Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys

350      360      370      380
*      *      *      *
CAA AAA ATC GTT TCT TAT TTG GAT TGG GAT AAA GGT GGA GGC
Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly

390      400      410      420      430
*      *      *      *      *
TCA GGC GGA G GGCCAAGTCGGCC ATCCCATATCAC GCGGCCGC GGATCC
Ser Gly Gly          SfiI                NotI      BamHI

```

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Figure 8C

```

1      10      20      30      40
*      *      *      *      *
AAGCTTTGCT AACATACTGC GTAATAAGGA GTCTTAATC ATG CCA GTT
HindIII                               Met Pro Val

50      60      70      80      90
*      *      *      *      *
CTT TTG GGT ATT CCG TTA TTA TTG CGT TTC CTC GGT TTC CTT
Leu Leu Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu

100     110     120     130
*      *      *      *
CTG GTA ACT TTG TTC GGC TAT CTG CTT ACT TTC CTT AAA AAG
Leu Val Thr Leu Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys

140     150     160     170
*      *      *      *
GGC TTC GGT AAG ATA GCT ATT GCT ATT TCA TTG TTT CTT GCT
Gly Phe Gly Lys Ile Ala Ile Ala Ile Ser Leu Phe Leu Ala

180     190     200     210
*      *      *      *
CTT ATT ATT GGG CTT AAC TCA ATT CTT GTG GGT TAT CTC TCT
Leu Ile Ile Gly Leu Asn Ser Ile Leu Val Gly Tyr Leu Ser

220     230     240     250
*      *      *      *
GAT ATT AGC GCA CAA TTA CCC TCT GAT TTT GTT CAG GGC GTT
Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe Val Gln Gly Val

260     270     280     290     300
*      *      *      *      *
CAG TTA ATT CTC CCG TCT AAT GCG CTT CCC TGT TTT TAT GTT
Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe Tyr Val

310     320     330     340
*      *      *      *
ATT CTC TCT GTA AAG GCT GCT ATT TTC ATT TTT GAC GTT AAA
Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys

350     360     370     380
*      *      *      *
CAA AAA ATC GTT TCT TAT TTG GAT TGG GAT AAA GGT GGA GGC
Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly

390     400     410     420     430
*      *      *      *      *
TCA GGC GGA TC GGCCAAGTCGGCC ATCCCATATCAC GCGGCCGC GGATCC
Ser Gly Gly      SfiI                      NotI      BamHI

```

Figure 9

18/51

```

      1          10          20          30          40
      *          *          *          *          *
    GAATTCGGG CTG GTW TCC TAC TGC AGT GGA AAA GCA ACG ATG
    EcoRI      Leu Val Ser Tyr Cys Ser Gly Lys Ala Thr Met

          50          60          70          80
          *          *          *          *
    CAG TGT GGT GAG AAT GAA AAG TAC GAT TCG TGC GGT AGC AAG
    Gln Cys Gly Glu Asn Glu Lys Tyr Asp Ser Cys Gly Ser Lys

          90          100          110          120
          *          *          *          *
    GAG TGC GAT AAG AAG TGC AAA TAT GAC GGA GTT GAG GAG GAA
    Glu Cys Asp Lys Lys Cys Lys Tyr Asp Gly Val Glu Glu Glu

          130          140          150          160
          *          *          *          *
    GAC GAC GAG GAA CCT AAT GTG CCA TGC CTA GTA CGT GTG TGT
    Asp Asp Glu Glu Pro Asn Val Pro Cys Leu Val Arg Val Cys

          170          180          190          200          210
          *          *          *          *          *
    CAT CAA GAT TGC GTA TGC GAA GAA GGA TTC TAT AGA AAC AAA
    His Gln Asp Cys Val Cys Glu Glu Gly Phe Tyr Arg Asn Lys

          220          230          240          250
          *          *          *          *
    GAT GAC AAA TGT GTA TCA GCA GAA GAC TGC GAA CTT GAC AAT
    Asp Asp Lys Cys Val Ser Ala Glu Asp Cys Glu Leu Asp Asn

          260          270          280          290
          *          *          *          *
    ATG GAC TTT ATA TAT CCC GGA ACT CGA AAC TGA ACGAAGGCTC
    Met Asp Phe Ile Tyr Pro Gly Thr Arg Asn

          300          310          320          330          340
          *          *          *          *          *
    CATTCTTGCT GCACAAGATC GATTGTCTCTCCCCTGCATCTCAGTAGTTTGC

          350          360          370          380          390          400
          *          *          *          *          *          *
    TACATTGTATATGGTAGCAAAAAATTAGCTTAGGGAGAATAAAATCTTTACCTAT

          410          420          430
          *          *          *
    ATTTAATCAATGAAGTATTCTCTTTCT poly(A)

```

Figure 10

19/51

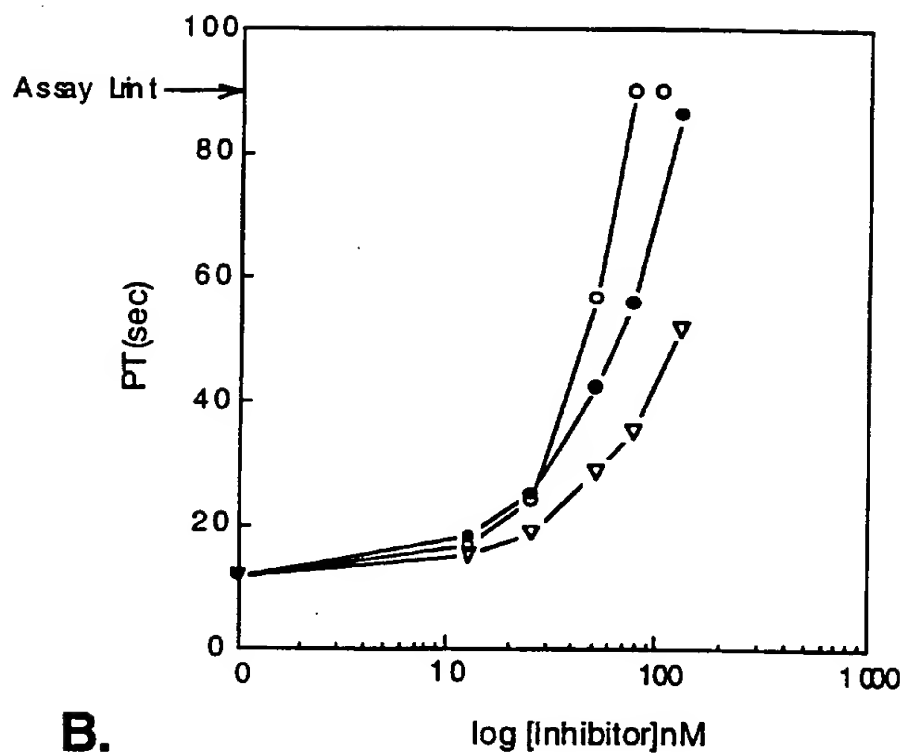
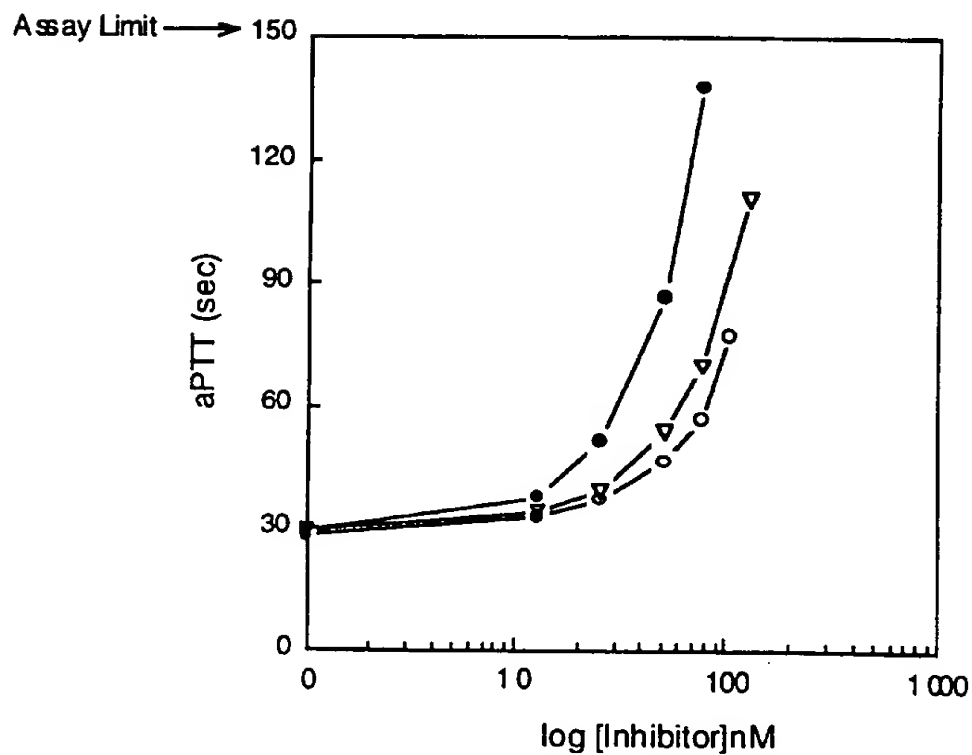
A.**B.**

Figure 11-1

NAP5	Met Lys Met Leu Tyr Ala Ile Ala Ile Met Phe Leu Leu Val
NAP6	Met Lys Met Leu Tyr Ala Ile Ala Ile Met Phe Leu Leu Val
NAPc2	Leu Val
AcNAP5	Met Arg Thr Leu Tyr Leu Ile Ser Ile Trp Leu Phe Leu Ile
AcNAP7	Met Ser Thr Leu Tyr Val Ile Ala Ile Cys Leu Leu Val
AcNAP4d1	Met Ala Val Leu Tyr Ser Val Ala Ile Ala Leu Leu Val
AcNAP4d2	
AduNAP4	
AduNAP7d1	Met Arg Met Leu Tyr Leu Val Pro Ile Trp Leu Leu Ile
AduNAP7d2	
HpoNAP5	Met Ile Arg Lys Leu Val Leu Leu Thr Ala Ile Val Thr

Figure 11-2

NAP5	Ser	Leu	Cys	Ser	Ala	Arg	Thr	Val	Arg	Lys	Ala	Tyr	Pro	Glu
NAP6	Ser	Leu	Cys	Ser	Thr	Arg	Thr	Val	Arg	Lys	Ala	Tyr	Pro	Glu
NAPc2	Ser	Tyr	Cys	Ser	Gly	---	---	---	---	Lys	Ala	Thr	Met	Gln
AceNAP5	Ser	Gln	Cys	Asn	Gly	---	---	---	---	Lys	Ala	Phe	Pro	Lys
AceNAP7	Ser	Gln	Cys	Asn	Gly	---	---	---	---	Arg	Thr	Val	Lys	Lys
AceNAP4d1	Ser	Gln	Cys	Ser	Gly	Lys	Pro	Asn	Asn	Val	Met	Thr	Asn	Ala
AceNAP4d2												Val	Pro	Ile
AduNAP4														Lys
AduNAP7d1	Ser	Leu	Cys	Ser	Gly	---	---	---	---	Lys	Ala	Ala	Lys	Lys
AduNAP7d2													Asp	Glu
HpoNAP5	Val	Val	Leu	Ser	Ala	---	---	---	---	---	---	---	Lys	Thr

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Figure 11-3

¹
 NAP5 Cys Gly Glu Asn Glu Trp Leu Asp Asp Cys Gly Thr Gln
²
 NAP6 Cys Gly Glu Asn Glu Trp Leu Asp Val Cys Gly Thr Lys
 NAPc2 Cys Gly Glu Asn Glu Lys Tyr Asp Ser Cys Gly Ser Lys
 AceNAP5 Cys Asp Val Asn Glu Arg Phe Glu Val Cys Gly Asn Leu
 AceNAP7 Cys Gly Lys Asn Glu Arg Tyr Asp Asp Cys Gly Asn Ala
 AceNAP4d1 Cys Gly Leu Asn Glu Tyr Phe Ala Glu Cys Gly Asn Met
 AceNAP4d2 Cys Gly Ser Asn Glu Arg Tyr Ser Asp Cys Gly Asn Asp
 AduNAP4 Cys Pro Thr Asp Glu Trp Phe Asp Trp Cys Gly Thr Tyr
 AduNAP7d1 Cys Gly Leu Asn Glu Arg Leu Asp --- Cys Gly Asn Leu
 AduNAP7d2 Cys Gly Pro Asp Glu Trp Phe Asp Tyr Cys Gly Asn Tyr
 HpoNAP5 Cys Gly Pro Asn Glu Glu Tyr Thr Glu Cys Gly Thr ---

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Figure 11-4

NAP5	Lys	Pro	³ Cys	Glu	Ala	Lys	⁴ Cys	---	---	---	---	Asn	Glu	Glu
NAP6	Lys	Pro	Cys	Glu	Ala	Lys	Cys	---	---	---	---	Ser	Glu	Glu
NAPc2	Glu	---	Cys	Asp	Lys	Lys	Cys	Lys	Tyr	Asp	Gly	Val	Glu	Glu
AceNAP5	Lys	Glu	Cys	Glu	Leu	Lys	Cys	---	---	---	---	---	---	---
AceNAP7	Lys	Asp	Cys	Glu	Thr	Lys	Cys	---	---	---	Gly	---	---	---
AceNAP4d1	Lys	Glu	Cys	Glu	His	Arg	Cys	Asn	Glu	Glu	Glu	Asn	Glu	Glu
AceNAP4d2	Lys	Gln	Cys	Glu	Arg	Lys	Cys	Asn	Glu	Asp	Asp	Tyr	Glu	Lys
AduNAP4	Lys	His	Cys	Glu	Leu	Lys	Cys	Asp	Arg	Glu	Leu	Thr	Glu	Lys
AduNAP7d1	Lys	Gln	Cys	Glu	Pro	Lys	Cys	Ser	Asp	Leu	Glu	Ser	Glu	Glu
AduNAP7d2	Lys	Lys	Cys	Glu	Arg	Lys	Cys	Ser	Glu	Glu	Thr	Ser	Glu	Lys
HpoNAP5	---	Pro	Cys	Glu	Pro	Lys	Cys	---	---	---	---	---	---	---

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Figure 11-5

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NAP5	Pro	Pro	Glu	Glu	Glu	Asp	Pro	Ile	---	---	⁵ Cys	Arg	Ser	Arg
NAP6	---	---	Glu	Glu	Glu	Asp	Pro	Ile	---	---	Cys	Arg	Ser	Phe
NAPc2	---	Glu	Asp	Asp	Glu	Glu	Pro	Asn	Val	Pro	Cys	Leu	Val	Arg
AceNAP5	---	---	Asp	Glu	Asp	Pro	Lys	Ile	---	---	Cys	---	Ser	Arg
AceNAP7	---	---	Glu	Glu	Glu	---	Lys	---	Val	---	Cys	Arg	Ser	Arg
AceNAP4d1	Arg	---	Asp	Glu	Glu	---	Arg	Ile	Thr	Ala	Cys	Leu	Ile	Arg
AceNAP4d2	Gly	---	Asp	Glu	---	---	---	---	---	Ala	Cys	Arg	Ser	His
AduNAP4	---	---	Glu	Glu	---	---	Gln	---	---	Ala	Cys	Leu	Ser	Arg
AduNAP7d1	Tyr	---	Glu	Glu	Glu	Asp	Glu	Ser	Lys	---	Cys	Arg	Ser	Arg
AduNAP7d2	Asn	---	Glu	Glu	---	---	---	---	---	Ala	Cys	Leu	Ser	Arg
HpoNAP5	---	---	Asn	Glu	Pro	Met	Pro	Asp	Ile	---	Cys	---	Thr	Leu

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Figure 11-6

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NAP5	Gly Cys ⁶	Leu	Pro	Pro	Ala Cys ⁷	Val Cys ⁸	Lys	Asp
NAP6	Ser Cys	Pro	Gly	Pro	Ala	Cys	Val	Cys
NAPc2	Val Cys	His	Gln	Asp	---	Cys	Val	Cys
AceNAP5	Ala Cys	Ile	Arg	Pro	Pro	Ala	Cys	Val
AceNAP7	Glu Cys	Thr	Ser	Pro	Gly	Ala	Cys	Val
AceNAP4d1	Val Cys	Phe	Arg	Pro	Gly	Ala	Cys	Val
AceNAP4d2	Val Cys	Glu	Arg	Pro	Gly	Ala	Cys	Val
AduNAP4	Val Cys	Glu	Lys	---	Ser	Ala	Cys	Val
AduNAP7d1	Glu Cys	Ser	Arg	Arg	---	Val	Cys	Val
AduNAP7d2	Ala Cys	Thr	Gly	Arg	---	Ala	Cys	Val
HpoNAP5	Asn Cys	Ile	Val	Asn	---	Val	Cys	Gln

Lys Asp
Glu Asp
Glu Glu
Asp Asp
Gln
Lys Asp
Glu Asp
Asn Asp
Glu
Lys Asp
Lys Pro

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Figure 11-7

NAP5 Gly Phe Tyr Arg Asp Thr Val Ile Gly Asp ⁹Cys Val Arg Glu
 NAP6 Gly Phe Tyr Arg Asp Thr Val Ile Gly Asp Cys Val Lys Glu
 NAPc2 Gly Phe Tyr Arg Asn Lys --- Asp Asp Lys Cys Val Ser Ala
 AceNAP5 Gly Phe Tyr Arg Asp Lys Tyr --- Gly Phe Cys Val Glu Glu
 AceNAP7 Gly Phe Tyr Arg Asp Pro Ala --- Gly Asp Cys Val Thr Asp
 AceNAP4d1 Gly Phe Tyr Arg Asn Arg Thr --- Gly Ser Cys Val Glu Glu
 AceNAP4d2 Gly Phe Tyr Arg Asn Lys Lys --- Gly Ser Cys Val Glu Ser
 AduNAP4 Gly Leu Tyr Arg Asp Lys Phe --- Gly Asn Cys Val Glu Lys
 AduNAP7d1 Gly Phe Tyr Arg Asn Lys Lys --- Gly Lys Cys Val Ala Lys
 AduNAP7d2 Gly Leu Tyr Arg Asp Asp Phe --- Gly Asn Cys Val Pro His
 HpoNAP5 Gly Phe Lys Arg Gly Pro Lys --- Gly --- Cys Val Ala Pro

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Figure 11-8

NAP5 Glu Glu --- Cys¹⁰ Asp Gln His --- --- Glu Ile Ile His
 NAP6 Glu Glu --- Cys Asp Gln His --- --- Glu Ile Ile His
 NAPc2 Glu Asp --- Cys Glu --- Leu Asp Asn Met Asp Phe Ile Tyr
 AceNAP5 Asp Glu --- Cys Asn Asp --- --- Met Glu Ile Ile Thr
 AceNAP7 Glu Glu --- Cys Asp Glu Trp Asn Asn Met Glu Ile Ile Thr
 AceNAP4d1 Asp Asp --- Cys Glu --- Tyr Glu Asn Met Glu Phe Ile Thr
 AceNAP4d2 Asp Asp --- Cys Glu --- Tyr Asp Asn Met Asp Phe Ile Thr
 AduNAP4 Asp Glu --- Cys Asn Asp --- --- Met Glu Ile Ile Thr
 AduNAP7d1 Asp Val --- Cys Glu Asp --- Asp Asn Met Glu Ile Ile Thr
 AduNAP7d2 Asp Glu --- Cys Asn Asp --- --- Met Glu Ile Ile Thr
 HpoNAP5 Gly Pro Gly Cys Lys end

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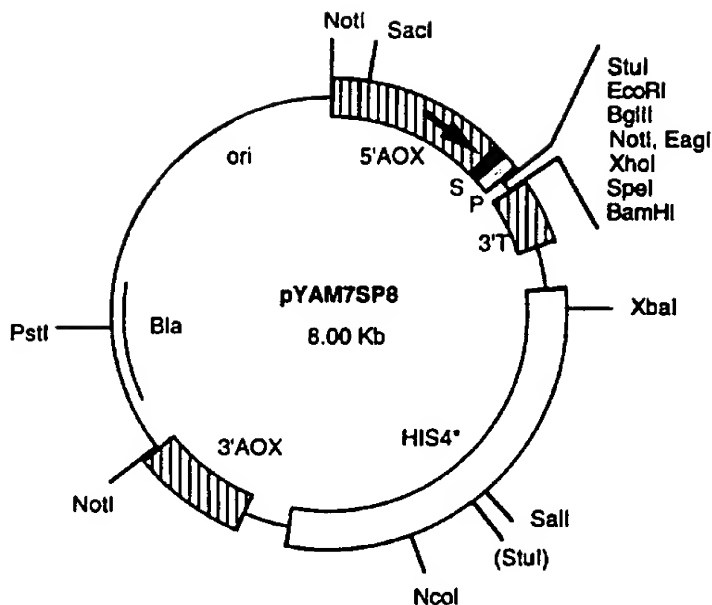
Figure 11-9

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NAP5	Val	end
NAP6	Val	end
NAPc2	Pro Gly Thr Arg Asn	end
AceNAP5	Phe Pro Pro Glu Thr Lys	end
AceNAP7	Met Pro Lys Gln	end
AceNAP4d1	Phe Ala Pro Glu	
AceNAP4d2	Phe Ala Pro Glu Thr Ser Arg	end
AduNAP4	Phe Ala Pro Glu Thr Lys	end
AduNAP7d1	Phe Pro Pro Glu	
AduNAP7d2	Phe Pro Pro Glu Thr Lys His	end
HpoNAP5		

Figur 12

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A**B**

<----5'AOX1-----><-----PHO1 secretion signal (S)--
TTATTCGAAACGATGTTCTCTCCAATTTTGTCTTGGAATTATTTTA

-----><-----Pro Sequence (P)-----
 GCTACTTTGCAATCTGTCTTCGCCCAGCCAGTTATCTCCACTACCGTTGGTTCC

-----><-----Multi-Cloning Site (MCS)
 GCTGCCGAGGGTTCTTTGGACAAGAGGCCTATCCGCGGAATTCAGATCTGAAT
 StuI SacII EcoRI BglII

-----><-----3'T----->
 GCGGCCGCTCGAGACTAGTGGATCCTTAGACA...
 NotI XhoI SpeI BamHI
 EagI

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Figure 13 A-1 (AcaNAP23)

```

          10          20          30          40
          *          *          *          *
GAATTCGCG GAATTCGCT TGCTACTACT CAACG ATG AAG ACG CTC
EcoRI                      Met Lys Thr Leu

50          60          70          80
*          *          *          *
TAT ATT GTC GCT ATA TGC TCG CTC CTC ATT TCG CTG TGT ACT
Tyr Ile Val Ala Ile Cys Ser Leu Leu Ile Ser Leu Cys Thr

90          100          110          120          130
*          *          *          *          *
GGA AAA CCT TCG GAG AAA GAA TGT GGT CCC CAT GAA AGA CTC
Gly Lys Pro Ser Glu Lys Glu Cys Gly Pro His Glu Arg Leu

          140          150          160          170
          *          *          *          *
GAC TGT GGC AAC AAG AAG CCA TGC GAG CGC AAG TGC AAA ATA
Asp Cys Gly Asn Lys Lys Pro Cys Glu Arg Lys Cys Lys Ile

          180          190          200          210
          *          *          *          *
GAG ACA AGT GAG GAG GAG GAT GAC TAC GAA GAG GGA ACC GAA
Glu Thr Ser Glu Glu Glu Asp Asp Tyr Glu Glu Gly Thr Glu

220          230          240          250
*          *          *          *
CGT TTT CGA TGC CTC TTA CGT GTG TGT GAT CAG CCT TAT GAA
Arg Phe Arg Cys Leu Leu Arg Val Cys Asp Gln Pro Tyr Glu

260          270          280          290
*          *          *          *
TGC ATA TGC GAT GAT GGA TAC TAC AGA AAC AAG AAA GGC GAA
Cys Ile Cys Asp Asp Gly Tyr Tyr Arg Asn Lys Lys Gly Glu

300          310          320          330          340
*          *          *          *          *
TGT GTG ACT GAT GAT GTA TGC CAG GAA GAC TTT ATG GAG TTT
Cys Val Thr Asp Asp Val Cys Gln Glu Asp Phe Met Glu Phe

          350          360          370          380
          *          *          *          *
ATT ACT TTC GCA CCA TAA ACCCAATAAT GACCAATGAC TCCCATTCTT
Ile Thr Phe Ala Pro

```

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Figure 13 A-2

```
390          400          410          420          430
  *          *          *          *          *
  CGTGATCAGC GTCGGTGGTT GACAGTCTCC CCTACATCTT AGTAGTTTTC
440          450          460          470          480
  *          *          *          *          *
  CTTGATAATG TATACATAAA CTGTACTTTC TGAGATAGAA TAAAGCTCTC

490
  *
  AACTAC poly(A)
```

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Figure 13 B-1 (AcaNAP24)

```

          10          20          30          40
          *          *          *          *
    GAATTCGCG GAATTCGCA ACG ATG AAG ACG CTC TAT ATT ATC
    EcoRI                      Met Lys Thr Leu Tyr Ile Ile

          50          60          70          80
          *          *          *          *
    GCT ATA TGC TCG CTC CTC ATT TCG TTG TGT ACT GGA AGA CCG
    Ala Ile Cys Ser Leu Leu Ile Ser Leu Cys Thr Gly Arg Pro

          90          100          110          120
          *          *          *          *
    GAA AAA AAG TGC GGT CCC GGT GAA AGA CTC GCC TGT GGC AAT
    Glu Lys Lys Cys Gly Pro Gly Glu Arg Leu Ala Cys Gly Asn

130          140          150          160          170
  *          *          *          *          *
    AAG AAG CCA TGC GAG CGC AAG TGC AAA ATA GAG ACA AGT GAG
    Lys Lys Pro Cys Glu Arg Lys Cys Lys Ile Glu Thr Ser Glu

          180          190          200          210
          *          *          *          *
    GAG GAG GAT GAC TAC CCA GAG GGA ACC GAA CGT TTT CGA TGC
    Glu Glu Asp Asp Tyr Pro Glu Gly Thr Glu Arg Phe Arg Cys

          220          230          240          250
          *          *          *          *
    CTC TTA CGT GTG TGT GAT CAG CCT TAT GAA TGC ATA TGC GAT
    Leu Leu Arg Val Cys Asp Gln Pro Tyr Glu Cys Ile Cys Asp

          260          270          280          290
          *          *          *          *
    GAT GGA TAC TAC AGA AAC AAG AAA GGC GAA TGT GTG ACT GAT
    Asp Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp

          300          310          320          330
          *          *          *          *
    GAT GTA TGC CAG GAA GAC TTT ATG GAG TTT ATT ACT TTC GCA
    Asp Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala

340          350          360          370          380
  *          *          *          *          *
    CCA TAA ACCCAATAAT GACCACTGGC TCCCATTCTT CGTGACCAGC
    Pro

```

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Figure 13 B-2

390	400	410	420	430
*	*	*	*	*
GTCGGTGGTT	GACAGTCTCC	CCTGCATCTT	AGTAGTTTTG	CTTGATAATG
440	450	460	470	
*	*	*	*	
TATCCATAAA	CAGTACTTTC	TGAGATAGAA	TAAAGCTCTC	AACT poly(A)

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Figure 13 C (AcaNAP25)

```

      10      20      30      40
      *      *      *      *
  GAATTCGTA CTACTCAACG ATG AAG ACG CTC TAT ATT ATC GCT
  EcoRI      Met Lys Thr Leu Tyr Ile Ile Ala

      50      60      70      80
      *      *      *      *
  ATA TGC TCG CTG CTC TTT TCA CTG TGT ACT GGA AGA CCG GAA
  Ile Cys Ser Leu Leu Phe Ser Leu Cys Thr Gly Arg Pro Glu

      90      100      110      120
      *      *      *      *
  AAA AAG TGC GGT CCC GGT GAA AGA CTC GAC TGT GCC AAC AAG
  Lys Lys Cys Gly Pro Gly Glu Arg Leu Asp Cys Ala Asn Lys

  130      140      150      160      170
  *      *      *      *      *
  AAG CCA TGC GAG CCC AAG TGC AAA ATA GAG ACA AGT GAG GAG
  Lys Pro Cys Glu Pro Lys Cys Lys Ile Glu Thr Ser Glu Glu

      180      190      200      210
      *      *      *      *
  GAG GAT GAC GAC GTA GAG GAT ACC GAT GTG AGA TGC CTC GTA
  Glu Asp Asp Asp Val Glu Asp Thr Asp Val Arg Cys Leu Val

      220      230      240      250
      *      *      *      *
  CGT GTG TGT GAA CGT CCT CTT AAA TGC ATA TGC AAG GAT GGA
  Arg Val Cys Glu Arg Pro Leu Lys Cys Ile Cys Lys Asp Gly

      260      270      280      290
      *      *      *      *
  TAC TAC AGA AAC AAG AAA GGC GAA TGT GTG ACT GAT GAT GTA
  Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp Asp Val

      300      310      320      330
      *      *      *      *
  TGC CAG GAA GAC TTT ATG GAG TTT ATT ACT TTC GCA CCA TAA
  Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro

  340      350      360      370      380
  *      *      *      *      *
  ACCCAATAAT GACCACTGGC TCCCATCTCTT CGTGATCAGC GTCGGTGGTT

  390      400      410      420      430
  *      *      *      *      *
  GACAGTCTCC CCTGCATCTT AGTTGCTTTG CTTGATAATC TATACATAAA

  440      450      460      470
  *      *      *      *
  CAGTACTTTC TGAGATAGAA TAAAGCTCTC AACT poly(A)

```

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Figure 13 D-1 (AcaNAP31)

```

      10      20      30      40      50
      *      *      *      *      *
GAATTC CGGA CTTACTAGTA CTCAGCGAAT CAAATACGAC TTACTACTAC
EcoRI

      60      70      80      90
      *      *      *      *
TCAACG ATG AAG ACG CTC TCT GCT ATC CCT ATA ATG CTG CTC
Met Lys Thr Leu Ser Ala Ile Pro Ile Met Leu Leu

     100     110     120     130
      *      *      *      *
CTG GTA TCG CAA TGC AGT GGA AAA TCA CTG TGG GAT CAG AAG
Leu Val Ser Gln Cys Ser Gly Lys Ser Leu Trp Asp Gln Lys

     140     150     160     170
      *      *      *      *
TGT GGT GAG AAT GAA AGG CTC GAC TGT GGC AAT CAG AAG GAC
Cys Gly Glu Asn Glu Arg Leu Asp Cys Gly Asn Gln Lys Asp

     180     190     200     210
      *      *      *      *
TGT GAG CGC AAG TGC GAT GAT AAA AGA AGT GAA GAA GAA ATT
Cys Glu Arg Lys Cys Asp Asp Lys Arg Ser Glu Glu Glu Ile

    220     230     240     250     260
      *      *      *      *      *
ATG CAG GCA TGT CTC ACA CGT CAA TGT CTT CCT CCT GTT TGC
Met Gln Ala Cys Leu Thr Arg Gln Cys Leu Pro Pro Val Cys

     270     280     290     300
      *      *      *      *
GTA TGT GAA GAT GGA TTC TAC AGA AAT GAC AAC GAC CAA TGT
Val Cys Glu Asp Gly Phe Tyr Arg Asn Asp Asn Asp Gln Cys

     310     320     330     340
      *      *      *      *
GTT GAT GAA GAA GAA TGC AAT ATG GAG TTT ATT ACT TTC GCA
Val Asp Glu Glu Glu Cys Asn Met Glu Phe Ile Thr Phe Ala

     350     360     370     380     390
      *      *      *      *      *
CCA TGA AGCAAATGAC AGCCGATGGT TTGGACTCTC GCTACAGATC
Pro

     400     410     420     430     440
      *      *      *      *      *
ACAGCTTTAC TGTTTCCCTT GCATCATAGT AGTTTGTGCTA GATAGTGTAT

```


Figure 13 E-1 (AcaNAP44)

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```

      10      20      30      40
      *      *      *      *
GAATTCGCG GAATTCGCA ACG ATG AAG ACG CTC TAT ATT ATC
EcoRI                      Met Lys Thr Leu Tyr Ile Ile

      50      60      70      80
      *      *      *      *
GCT ATA TGC TCG CTC CTC ATT TCG CTG TGT ACT GGA AGA CCG
Ala Ile Cys Ser Leu Leu Ile Ser Leu Cys Thr Gly Arg Pro

      90      100      110      120
      *      *      *      *
GAA AAA AAG TGC GGT CCC GGT GAA AGA CTC GAC TGT GCC AAC
Glu Lys Lys Cys Gly Pro Gly Glu Arg Leu Asp Cys Ala Asn

130      140      150      160      170
*      *      *      *      *
AAG AAG CCA TGC GAG CCC AAG TGC AAA ATA GAG ACA AGT GAG
Lys Lys Pro Cys Glu Pro Lys Cys Lys Ile Glu Thr Ser Glu

      180      190      200      210
      *      *      *      *
GAG GAG GAT GAC GAC GTA GAG GAA ACC GAT GTG AGA TGC CTC
Glu Glu Asp Asp Asp Val Glu Glu Thr Asp Val Arg Cys Leu

      220      230      240      250
      *      *      *      *
GTA CGT GTG TGT GAA CGG CCT CTT AAA TGC ATA TGC AAG GAT
Val Arg Val Cys Glu Arg Pro Leu Lys Cys Ile Cys Lys Asp

      260      270      280      290
      *      *      *      *
GGA TAC TAC AGA AAC AAG AAA GGC GAA TGT GTG ACT GAT GAT
Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp Asp

      300      310      320      330
      *      *      *      *
GTA TGC CAG GAA GAC TTT ATG GAG TTT ATT ACT TTC GCA CCA
Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro

340      350      360      370      380
*      *      *      *      *
TAA ACCCAATAAT GACCACTGGC TCCCATTCTT CGTGATCAGC

      390      400      410      420      430
      *      *      *      *      *
GTCGGTGGTT GACAGTCTCC CCTGCATCTT AGTTGCTTTG CTTGATAATC

```

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Figure 13 E-2

440	450	460	470
*	*	*	*
TATACATAAA	CAGTACTTTC	TGAGATAGAA	TAAAGCTCTC
AACTAC			

poly(A)

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Figure 13 F-1 (AcaNAP45)

10	20	30	40
* GAATTCGGA	* AAA	* ATG	* CTG
EcoRI	Met	Leu	Met
50	60	70	80
* TTG	* CTA	* CTC	* ATT
Leu	Leu	Leu	Ile
90	100	110	120
* TGT	* GGT	* CTC	* AAT
Cys	Gly	Leu	Asn
130	140	150	160
* TGC	* GAG	* AAA	* AAG
Cys	Glu	Lys	Lys
170	180	190	200
* AAG	* GAG	* GAA	* GAT
Lys	Glu	Glu	Asp
220	230	240	250
* CGT	* CGT	* GTT	* TGT
Arg	Arg	Val	Cys
260	270	280	290
* AAG	* GGC	* CAA	* TGT
Lys	Gly	Gln	Cys
300	310	320	330
* ATG	* GAG	* ATT	* ATC
Met	Glu	Ile	Ile
340	350	360	370
* GAT	* GAA	* TGG	* TTC
Asp	Glu	Trp	Phe
380	390	400	410
* CGC	* AAG	* TGC	* AAT
Arg	Lys	Cys	Asn
420			
* GAG			
Glu			

Figure 13 F-2

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430 440 450 460
* * * *

TGC CTC TCA CGT GCT TGT ACT GGT CGT GCT TGT GTT TGC AAC
Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys Asn

470 480 490 500
* * * *

GAC GGA CTG TAC AGA GAC GAT TTT GGC AAT TGT GTT GAG AAA
Asp Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Glu Lys

510 520 530 540
* * * *

GAC GAA TGT AAC GAT ATG GAG ATT ATC ACT TTT CCA CCG GAA
Asp Glu Cys Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu

550 560 570 580
* * * *

ACC AAA CAC TGA CCAAAGGCTC TAACTCTCGC TACATAACGT
Thr Lys His

590 600 610 620 630
* * * *

CAGTGCTTGA ATTGCCCTT TACGAGTTAG TAATTTTGAC TAACTCTGTG

640 650 660 670 680
* * * *

TAATTGAGCA TTGTCTACTG ATGGTGAAAA TGAAGTGTTT AATGTCT

poly(A)

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Figure 13 G-1 (AcaNAP47)

```

          10          20          30          40
          *          *          *          *
    GAATTCGCG GAATTCGGT TGGCGGCAGA AAA ATG CTG ATG CTC
    EcoRI                               Met Leu Met Leu

          50          60          70          80
          *          *          *          *
    TAC CTT GTT CCT ATC TGG TTC CTG CTC ATT TCG CAA TGC AGT
    Tyr Leu Val Pro Ile Trp Phe Leu Leu Ile Ser Gln Cys Ser

          90          100          110          120
          *          *          *          *
    GGA AAA TCC GCG AAG AAA TGT GGC CTC AAT GAA AAA TTG GAC
    Gly Lys Ser Ala Lys Lys Cys Gly Leu Asn Glu Lys Leu Asp

130          140          150          160          170
  *          *          *          *          *
    TGT GGC AAT CTG AAG GCA TGC GAG AAA AAG TGC AGC GAC TTG
    Cys Gly Asn Leu Lys Ala Cys Glu Lys Lys Cys Ser Asp Leu

          180          190          200          210
          *          *          *          *
    GAC AAT GAG GAG GAT TAT GGG GAG GAA GAT GAG TCG AAA TGC
    Asp Asn Glu Glu Asp Tyr Gly Glu Glu Asp Glu Ser Lys Cys

          220          230          240          250
          *          *          *          *
    CGA TCA CGT GAA TGT ATT GGT CGT GTT TGC GTA TGC GAT GAA
    Arg Ser Arg Glu Cys Ile Gly Arg Val Cys Val Cys Asp Glu

          260          270          280          290
          *          *          *          *
    GGA TTC TAC AGA AAC AAG AAG GGC CAA TGT GTG ACA AGA GAC
    Gly Phe Tyr Arg Asn Lys Lys Gly Gln Cys Val Thr Arg Asp

          300          310          320          330
          *          *          *          *
    GAT TGC GAG TAT GAC AAT ATG GAG ATT ATC ACT TTT CCA CCA
    Asp Cys Glu Tyr Asp Asn Met Glu Ile Ile Thr Phe Pro Pro

          340          350          360          370          380
          *          *          *          *          *
    GAA GAT AAA TGT GGT CCC GAT GAA TGG TTC GAC TGG TGT GGA
    Glu Asp Lys Cys Gly Pro Asp Glu Trp Phe Asp Trp Cys Gly

          390          400          410          420
          *          *          *          *
    ACT TAC AAG CAG TGT GAG CGC AAG TGC AGT GAG GAG CTA AGT
    Thr Tyr Lys Gln Cys Glu Arg Lys Cys Ser Glu Glu Leu Ser
  
```

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Figure 13 G-2

430 440 450 460
 * * * *
 GAG AAA AAT GAG GAG GCA TGC CTC TCA CGT GCT TGT ACT GGT
 Glu Lys Asn Glu Glu Ala Cys Leu Ser Arg Ala Cys Thr Gly

 470 480 490 500
 * * * *
 CGT GCT TGC GTT TGC AAC GAC GGA TTG TAT AGA GAC GAT TTT
 Arg Ala Cys Val Cys Asn Asp Gly Leu Tyr Arg Asp Asp Phe

 510 520 530 540
 * * * *
 GGC AAT TGT GTT GAG AAA GAC GAA TGT AAC GAT ATG GAG ATT
 Gly Asn Cys Val Glu Lys Asp Glu Cys Asn Asp Met Glu Ile

 550 560 570 580
 * * * *
 ATC ACT TTT CCA CCG GAA ACC AAA CAC TGA CCAAAGGCTC
 Ile Thr Phe Pro Pro Glu Thr Lys His

 590 600 610 620 630
 * * * * *
 TAGCTCTCGC TACATAACGT CAGTGCTTGA ATTGTCCCTT TACGTGTTAG

 640 650 660 670 680
 * * * * *
 TAATTTTGAC TAACTCTGTG TATTTGAGCA TTGTCTACTA ATGGTGAAAA

 690 700
 * *
 TGAAGCTTTT CAATGACT poly(A)

Figure 13 H-1 (AcaNAP48)

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```

      10      20      30      40
      *      *      *      *
    GAATTCGTA CGACCTACTA CTA CTCAACG ATG AAG GCG CTC TAT
    EcoRI                      Met Lys Ala Leu Tyr

      50      60      70      80
      *      *      *      *
    GTT ATC TCT ATA ACG TTG CTC CTG GTA TGG CAA TGC AGT GCA
    Val Ile Ser Ile Thr Leu Leu Leu Val Trp Gln Cys Ser Ala

      90      100      110      120
      *      *      *      *
    AGA ACA GCG AGG AAA CCC CCA ACG TGT GGT GAA AAT GAA AGG
    Arg Thr Ala Arg Lys Pro Pro Thr Cys Gly Glu Asn Glu Arg

    130      140      150      160      170
    *      *      *      *      *
    GTC GAA TGG TGT GGC AAG CAG TGC GAG ATC ACA TGT GAC GAC
    Val Glu Trp Cys Gly Lys Gln Cys Glu Ile Thr Cys Asp Asp

      180      190      200      210
      *      *      *      *
    CCA GAT AAG ATA TGC CGC TCA CTC GCT TGT CCT GGT CCT CCT
    Pro Asp Lys Ile Cys Arg Ser Leu Ala Cys Pro Gly Pro Pro

      220      230      240      250
      *      *      *      *
    GCT TGC GTA TGC GAC GAC GGA TAC TAC AGA GAC ACG AAC GTT
    Ala Cys Val Cys Asp Asp Gly Tyr Tyr Arg Asp Thr Asn Val

      260      270      280      290
      *      *      *      *
    GGC TTG TGT GTA CAA TAT GAC GAA TGC AAC GAT ATG GAT ATT
    Gly Leu Cys Val Gln Tyr Asp Glu Cys Asn Asp Met Asp Ile

    300      310      320      330      340
    *      *      *      *      *
    ATT ATG GTT TCA TAG GGTGACTGA AGAATCGAAC AACCGGTGCA
    Ile Met Val Ser

      350      360      370      380      390
      *      *      *      *      *
    CAACTTCTAT GCTTGACTAT CTCTCTTGCA TCATGCAAGT TTAGCTAGAT

      400      410      420      430      440
      *      *      *      *      *
    AGTGTATATA TTAGCAAGAC CCCTTGGGGA GAATGAAGCT TCCCAACTAT

      450      460      470      480      490
      *      *      *      *      *
    ATTAAATCAA TAACGTTTTT GCTTCATGTA CACGTGCTCA GCACATTCAT

```

Figure 13 H-2

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500 510 520
* * *
ATCCA^{CT}CTCCT CACA^{CT}TCCAT GAAAGCAGTG AAATGTT poly(A)

Figure 14

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```

      10      20      30      40
      *      *      *      *
GCC AAC TCT TCG AAC ATG ATT CGA GGC CTC GTT CTT CTT TCT CTC CTG
                      Met Ile Arg Gly Leu Val Leu Leu Ser Leu Leu>

      50      60      70      80      90
      *      *      *      *      *
TTT TGC GTC ACT TTT GCA GCG AAG AGA GAT TGT CCA GCA AAT GAG GAA
Phe Cys Val Thr Phe Ala Ala Lys Arg Asp Cys Pro Ala Asn Glu Glu>

      100      110      120      130      140
      *      *      *      *      *
TGG AGG GAA TGT GGC ACT CCA TGT GAA CCA AAA TGC AAT CAA CCG ATG
Trp Arg Glu Cys Gly Thr Pro Cys Glu Pro Lys Cys Asn Gln Pro Met>

      150      160      170      180      190
      *      *      *      *      *
CCA GAT ATA TGT ACT ATG AAT TGT ATC GTC GAT GTG TGT CAA TGC AAG
Pro Asp Ile Cys Thr Met Asn Cys Ile Val Asp Val Cys Gln Cys Lys>

      200      210      220      230      240
      *      *      *      *      *
GAG GGA TAC AAG CGT CAT GAA ACG AAG GGA TGC TTA AAG GAA GGA TCA
Glu Gly Tyr Lys Arg His Glu Thr Lys Gly Cys Leu Lys Glu Gly Ser>

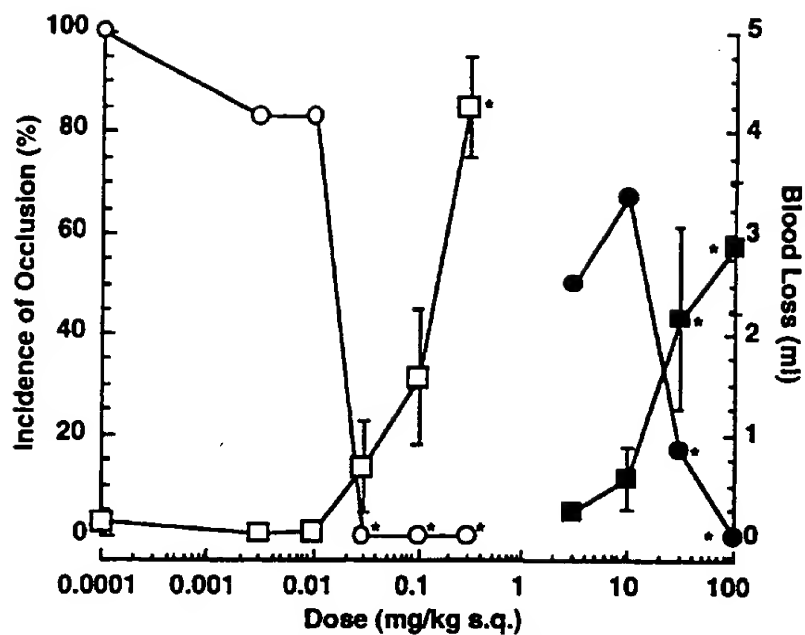
      250      260      270      280
      *      *      *      *
GCT GAT TGT AAA TAA GTT ATC AGA ACG CTC GTT TTG TCT TAC ATT AGA
Ala Asp Cys Lys ***

      290      300      310      320      330
      *      *      *      *      *
TGG GTG AGC TGA TGT ATC TGT CAG ATA AAC TCT TTC TTC TAA AAA AAA

      340      350      360
      *      *      *
AAA AAA AAA AAA AAA AAA AAA AAA A

```

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FIGURE 15

Efficacy:	Bleeding:	Estimated 3.5 hr Blood Loss @:	
		ED ₅₀	ED ₁₀₀
○ NAP-5	□ NAP-5	0.3	0.7
● LMWH	■ LMWH	1.2	2.9

* p<0.05 vs saline

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FIGURE 16

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10												
AcaNAP5	KAYPQGE	NEMLDQC	GTQKP	CEAKC	NEEPPE	EE	DPIC	RS	RGCL	LPP	ACVCK	D	GFYRD	TV	IGDCVR	E	EDCDQ	H	EIIHV			
AcaNAP6	KAYPQGE	NEMLDVC	GTQKP	CEAKC	SEEE	EE	DPIC	RS	FSCP	GPA	ACVCE	D	GFYRD	TV	IGDCVK	E	EDCDQ	H	EIIHV			
AcaNAP48	RTARKPPTQGE	NERVEMC	G	KQ	CEITC	DOP	DKIC	RS	LACP	GPP	ACVCD	D	GYTRD	TN	VGLCVQ	Y	DEBND		MDIIMVS			
AcaNAP23	KPSEKQGP	HERLD	C	GNKKP	CERKC	KIETSEEDDYEDQTE	RPRC	LL	RVCD	QPY	ECICD	D	GYTRN	K	KGBCVT	D	DVCQE		DFMEFITFAP			
AcaNAP24	RPEKQGP	GERLD	C	GNKKP	CERKC	KIETSEEDDYEDQTE	RPRC	LL	RVCD	QPY	ECICD	D	GYTRN	K	KGBCVT	D	DVCQE		DFMEFITFAP			
AcaNAP25	RPEKQGP	GERLD	C	GNKKP	CEPKC	KIETSEEDDYEDQTE	DT	DVRC	LV	RVCE	RPL	KCICK	D	GYTRN	K	KGBCVT	D	DVCQE		DFMEFITFAP		
AcaNAP44	RPEKQGP	GERLD	C	GNKKP	CEPKC	KIETSEEDDYEDQTE	ET	DVRC	LV	RVCE	RPL	KCICK	D	GYTRN	K	KGBCVT	D	DVCQE		DFMEFITFAP		
AcaNAP31, 42, 46	KSLWDQKQGE	NERLD	C	GNQKD	CERKC	DKRSEE	EI	MQAC	LT	RQCL	PP	VCVCE	D	GFYRN	D	NDQCVD	E	EDCN		MEFITFAP		
AcaNAP4-d1	KPRNVMNACGL	NEYFABC	GNHKE	CEHRC	NEE	ENEERDE	ER	ITAC	LI	RVCF	RPG	ACVCK	D	GFYRN	R	TGSCVE	E	DOCE		YENMEFITFAPE-->		
AcaNAP4-d2	VPIQGS	NERYSDC	GNKQK	CERKC	NED	DYKNG	DEAC	RS	HVCE	RPG	ACVCE	D	GFYRN	K	RGSCVE	S	DOCE		YANNDFITFAPE-->			
AcaNAP45d1	KSARKQGL	NEKLD	C	GNLKA	CEKGC	SDL	INEEDYKE	ED	ESKC	RS	RECSR	R	VCVCD	E	GFYRN	K	KQCVT	R	DOCEY		DNMEITFPPE-->	
AcaNAP47d1	KSARKQGL	NEKLD	C	GNLKA	CEKGC	SDL	INEEDYGB	ED	ESKC	RS	RECIIG	R	VCVCD	E	GFYRN	K	KQCVT	R	DOCEY		DNMEITFPPE-->	
AcaNAP7-d1	KAARKQGL	NEKLD	C	GNLKA	CEPKC	SDL	ESEYEE	ED	ESKC	RS	REBS	R	VCVCD	E	GFYRN	K	KQCVT	R	DOCEY		DNMEITFPPE-->	
AcaNAP45d2	DKQGP	DEMFDMC	GTQKQ	CERKC	NKE	LSEKD	EEAC	LS	RACTG	R	ACVCH	D	GLYRD	D	FGNCVE	K	DEBND		MEITFPPE-->		MEITFPPE-->	
AcaNAP47d2	DKQGP	DEMFDMC	GTQKQ	CERKC	SEE	LSEKN	EEAC	LS	RACTG	R	ACVCH	D	GLYRD	D	FGNCVE	K	DEBND		MEITFPPE-->		MEITFPPE-->	
AduNAP4	KCPT	DEMFDMC	GTQKH	CEKGC	DRE	LTEKE	EEAC	LS	RVCE	K	S	ACVCH	D	GLYRD	K	FGNCVE	K	DEBND		MEITFPPE-->		MEITFPPE-->
AduNAP7-d2	DEQGP	DEMFDMC	GTQKH	CEKGC	SEE	TSEKN	EEAC	LS	RACT	G	R	ACVCH	D	GLYRD	D	FGNCVP	H	DEBND		MEITFPPE-->		MEITFPPE-->
AcaNAP5	KAPFKQDV	NERFEVC	GNLKE	CEKGC	D		ED	PAIC	S	RACI	RPP	ACVCD	D	GFYRD	K	YGFVCE	E	DEBND		MEITFPPE-->		MEITFPPE-->
AcaNAP7	RTVKQKQK	NERYDDC	GNAND	CETKQ	G		EE	EVKC	RS	REBT	SRG	ACVCE	Q	GFYRD	P	AGDCVT	D	EBEDE		WNNMEITMPKQ		WNNMEITMPKQ
AcaNAPc2	KATMQQGE	NEKYDSC	GSKE	CDKKG	KYDGVVEEDDE	EP	NVPC	LV	RVCH	Q	DCVCE	E	GFYRN	K	DOKCVS	A	EDCEL		DNMDFIYVGTNR		DNMDFIYVGTNR	
HpoNAP5	KTCGP	NEEYTBG	GTP	CEPKC	NERMPDI		C	TLN	CI	VNV	COCK	P	GFYRGPKG		CVA	RGKQ	K					
NamNAP	KRDGPA	NEEMRDC	GTP	CEPKC	NQMPDI		C	TMN	CI	VDV	COCK	E	GYRHEHKG		CLKQSSADC	K						

NAP = nematode anticoagulant protein

Aca = *Ancylostoma caninum*
 Ace = *Ancylostoma ceylanium*
 Adu = *Ancylostoma duodenale*
 Hpo = *Heligmosmoides polygyrus*
 Asu = *Ascaris suum*
 Nam = *Necator americanus*

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Figure 17

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Lys Pro Asn Asn Val Met Thr Asn Ala **Cys** Gly Leu Asn Glu
 1 5 10
 Tyr Phe Ala Glu **Cys** Gly Asn Met Lys Glu **Cys** Glu His Arg
 15 20 25
Cys Asn Glu Glu Glu Asn Glu Glu Arg Asp Glu Glu Arg Ile
 30 35 40
 Thr Ala **Cys** Leu Ile Arg Val **Cys** Phe Arg Pro Gly Ala **Cys**
 45 50 55
 Val **Cys** Lys Asp Gly Phe Tyr Arg Asn Arg Thr Gly Ser **Cys**
 60 65 70
 Val Glu Glu Asp Asp **Cys** Glu Tyr Glu Asn Met Glu Phe Ile
 75 80
 Thr Phe Ala Pro Glu Val Pro Ile **Cys** Gly Ser Asn Glu Arg
 85 90 95
 Tyr Ser Asp **Cys** Gly Asn Asp Lys Gln **Cys** Glu Arg Lys **Cys**
 100 105 110
 Asn Glu Asp Asp Tyr Glu Lys Gly Asp Glu Ala **Cys** Arg Ser
 115 120 125
 His Val **Cys** Glu Arg Pro Gly Ala **Cys** Val **Cys** Glu Asp Gly
 130 135 140
 Phe Tyr Arg Asn Lys Lys Gly Ser **Cys** Val Glu Ser Asp Asp
 145 150
Cys Glu Tyr Asp Asn Met Asp Phe Ile Thr Phe Ala Pro Glu
 155 160 165
 Thr Ser Arg
 170

Figure 18

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Lys Ser Ala Lys Lys **Cys** Gly Leu Asn Glu Lys Leu Asp **Cys**
 1 5 10
 Gly Asn Leu Lys Ala **Cys** Glu Lys Lys **Cys** Ser Asp Leu Asp
 15 20 25
 Asn Glu Glu Asp Tyr Lys Glu Glu Asp Glu Ser Lys **Cys** Arg
 30 35 40
 Ser Arg Glu **Cys** Ser Arg Arg Val **Cys** Val **Cys** Asp Glu Gly
 45 50 55
 Phe Tyr Arg Asn Lys Lys Gly Gln **Cys** Val Thr Arg Asp Asp
 60 65 70
Cys Glu Tyr Asp Asn Met Glu Ile Ile Thr Phe Pro Pro Glu
 75 80
 Asp Lys **Cys** Gly Pro Asp Glu Trp Phe Asp Trp **Cys** Gly Thr
 85 90 95
 Tyr Lys Gln **Cys** Glu Arg Lys **Cys** Asn Lys Glu Leu Ser Glu
 100 105 110
 Lys Asp Glu Glu Ala **Cys** Leu Ser Arg Ala **Cys** Thr Gly Arg
 115 120 125
 Ala **Cys** Val **Cys** Asn Asp Gly Leu Tyr Arg Asp Asp Phe Gly
 130 135 140
 Asn **Cys** Val Glu Lys Asp Glu **Cys** Asn Asp Met Glu Ile Ile
 145 150
 Thr Phe Pro Pro Glu Thr Lys His
 155 160

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Figure 19

Lys Ser Ala Lys Lys **Cys** Gly Leu Asn Glu Lys Leu Asp **Cys**
 1 5 10
 Gly Asn Leu Lys Ala **Cys** Glu Lys Lys **Cys** Ser Asp Leu Asp
 15 20 25
 Asn Glu Glu Asp Tyr Gly Glu Glu Asp Glu Ser Lys **Cys** Arg
 30 35 40
 Ser Arg Glu **Cys** Ile Gly Arg Val **Cys** Val **Cys** Asp Glu Gly
 45 50 55
 Phe Tyr Arg Asn Lys Lys Gly Gln **Cys** Val Thr Arg Asp Asp
 60 65 70
Cys Glu Tyr Asp Asn Met Glu Ile Ile Thr Phe Pro Pro Glu
 75 80
 Asp Lys **Cys** Gly Pro Asp Glu Trp Phe Asp Trp **Cys** Gly Thr
 85 90 95
 Tyr Lys Gln **Cys** Glu Arg Lys **Cys** Ser Glu Glu Leu Ser Glu
 100 105 110
 Lys Asn Glu Glu Ala **Cys** Leu Ser Arg Ala **Cys** Thr Gly Arg
 115 120 125
 Ala **Cys** Val **Cys** Asn Asp Gly Leu Tyr Arg Asp Asp Phe Gly
 130 135 140
 Asn **Cys** Val Glu Lys Asp Glu **Cys** Asn Asp Met Glu Ile Ile
 145 150
 Thr Phe Pro Pro Glu Thr Lys His
 155 160

Figure 20

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Lys Ala Ala Lys Lys **Cys** Gly Leu Asn Glu Arg Leu Asp **Cys**
 1 5 10
 Gly Asn Leu Lys Gln **Cys** Glu Pro Lys **Cys** Ser Asp Leu Glu
 15 20 25
 Ser Glu Glu Tyr Glu Glu Glu Asp Glu Ser Lys **Cys** Arg Ser
 30 35 40
 Arg Glu **Cys** Ser Arg Arg Val **Cys** Val **Cys** Asp Glu Gly Phe
 45 50 55
 Tyr Arg Asn Lys Lys Gly Lys **Cys** Val Ala Lys Asp Val **Cys**
 60 65 70
 Glu Asp Asp Asn Met Glu Ile Ile Thr Phe Pro Pro Glu Asp
 75 80
 Glu **Cys** Gly Pro Asp Glu Trp Phe Asp Tyr **Cys** Gly Asn Tyr
 85 90 95
 Lys Lys **Cys** Glu Arg Lys **Cys** Ser Glu Glu Thr Ser Glu Lys
 100 105 110
 Asn Glu Glu Ala **Cys** Leu Ser Arg Ala **Cys** Thr Gly Arg Ala
 115 120 125
Cys Val **Cys** Lys Asp Gly Leu Tyr Arg Asp Asp Phe Gly Asn
 130 135 140
Cys Val Pro His Asp Glu **Cys** Asn Asp Met Glu Ile Ile Thr
 145 150
 Phe Pro Pro Glu Thr Lys His
 155 160



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(71) Applicant (for all designated States except US): CORVAS INTERNATIONAL, INC. [US/US]; 3030 Science Park Road, San Diego, CA 92121 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).
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(54) Title: NEMATODE-EXTRACTED SERINE PROTEASE INHIBITORS AND ANTICOAGULANT PROTEINS

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10		
AcaNAP5	KAYPECGE	NEWLDGC	GTOKP	CEAKC	NEEPPPE	EE DPIC	RS RCCL	LPP ACVCK	D GFYRD	TV IGDCVR	E EECDO	H EIIHV
AcaNAP6	KAYPECGE	NEWLDVC	GTKKP	CEAKC	SEEE	EE DPIC	RS FSCP	GPA ACVCE	D GFYRD	TV IGDCVK	E EECDO	H EIIHV
AcaNAP48	RTARKPPTCGE	NERVEWC	G KO CEITC	DDP	DKIC	RS LACP	GPP ACVCD	D GYYRD	TN VGLCVO	Y DECND	MDIIMVS	
AcaNAP23	KPSEKECGP	GERLD	C GNKKP	CERKC	KIETSEEDDYE	EGTE	RFRC LL RVCD	OPY ECICD	D GYYRN	K KGECVT	D DVCQE	DFMEFITFAP
AcaNAP24	RPEKKCGP	GERLA	C GNKKP	CERKC	KIETSEEDDYP	EGTE	RFRC LL RVCD	OPY ECICD	D GYYRN	K KGECVT	D DVCQE	DFMEFITFAP
AcaNAP25	RPEKKCGP	GERLD	C ANKKP	CEPKC	KIETSEEDDOVE	DT DVRC	LV RVCE	RPL KCICK	D GYYRN	K KGECVT	D DVCQE	DFMEFITFAP
AcaNAP44	RPEKKCGP	GERLD	C ANKKP	CEPKC	KIETSEEDDOVE	DT DVRC	LV RVCE	RPL KCICK	D GYYRN	K KGECVT	D DVCQE	DFMEFITFAP
AcaNAP31, 42, 46	KSLWQCKGE	NERLD	C GNQKD	CERKC	DKRSEE	EI MQAC	LT ROCL	PP VCVCE	D GFYRN	D NDQCV	E EECN	DFMEFITFAP
AcaNAP4-d1	KPNVMTNACGL	NEYFAEC	GNPKE	CEHRC	NEE ENEERDE	ER ITAC	LI RVCF	RPG ACVCK	D GFYRN	K TGSVE	E DDCCE	YENMEFITFAPE+
AcaNAP4-d2	VPICGS	NERYSOC	GNDQK	CERKC	NED DYKKG	DEAC	RS HVCE	RPG ACVCE	D GFYRN	K KGSCVE	S DDCCE	YENMEFITFAPE+
AcaNAP45d1	KSAAKCGL	NEKLD	C GNLKA	CEKCC	SDL DNEEDYKE	ED ESKC	RS RECSR	R VCVCD	E GFYRN	K KGOCVT	R DDCCE	DNMEIITFPPE+
AcaNAP47d1	KSAAKCGL	NEKLD	C GNLKA	CEKCC	SDL DNEEDYGE	ED ESKC	RS RECIG	R VCVCD	E GFYRN	K KGOCVT	R DDCCE	DNMEIITFPPE+
AduNAP7-d1	KAACKCGL	NERLD	C GNKKP	CEPKC	SDL ESEEE	ED ESKC	RS RECS	R VCVCD	E GFYRN	K KGKCV	K DVCDE	DNMEIITFPPE+
AcaNAP45d2	DKCGP	DEWFDWC	GTYYKQ	CERKC	NKE LSEKD	EEAC	LS RACTG	R ACVCN	D GLYRD	D FGNCVE	K DECND	HEIITFPETKH
AcaNAP47d2	DKCGP	DEWFDWC	GTYYKQ	CERKC	SEE LSEKN	EEAC	LS RACTG	R ACVCN	D GLYRD	D FGNCVE	K DECND	HEIITFPETKH
AduNAP4	KCPPT	DEWFDWC	GTYYKH	CEKCC	DRE LTEKE	EEAC	LS RVCE	K S ACVCN	D GLYRD	K FGNCVE	K DECND	HEIITFAPEETK
AduNAP7-d2	DECGP	DEWFDYC	GNYYKH	CERKC	SEE TSEKN	EEAC	LS RACT	G R ACVCK	D GLYRD	D FGNCVP	H DECND	HEIITFPETKH
AceNAP5	KAFPKCDV	NERFEVC	GNLKE	CEKCC	D	ED PKIC	S RACI	RPP ACVCD	D GFYRD	K YGFCVE	E DECND	HEIITFPETKH
AceNAP7	RTVKKCGK	NERYDDC	GNADK	CEKCC	G	EE EKVC	RS RECT	SPG ACVCE	D GFYRD	P AGDCVT	D EECDE	WNHMEIITHPKQ
AcaNAPc2	KATMOGGE	NEKYDSC	GSKE	CDKCC	KYDGVEEEDDE	EP NVPC	LV RVCH	Q DCVCE	E GFYRN	K DOKCVS	A EDCCL	DNNOFITPYGTRN
HpoNAP5	KTCGP	NEEYTEC	GTP	CEPKC	NEPMPDI	C TLN	CI VNV	COCK	P GFKRGP	K CVA	PGPGC	K
NamNAP	KROCPA	NEEWREC	GTP	CEPKC	NOPMPI	C TMN	CI VDV	COCK	E GYKRHETK	G	CLKEGSAD	K

(57) Abstract

Proteins which have activity as anticoagulants and/or serine protease inhibitors and have at least one NAP domain are described. Certain of these proteins have factor Xa inhibitory activity and others have activity as inhibitors of factor VIIa/TF. These proteins can be isolated from natural sources as nematodes, chemically synthesized or made by recombinant methods using various DNA expression systems.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/13231

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/15 C07K14/81 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, no. 13, 20 June 1995, WASHINGTON US, pages 6152-6156, XP000512152 M. CAPELLO ET AL.: "Ancylostoma caninum anticoagulant peptide: A hookworm derived inhibitor of human coagulation factor Xa" see page 6152, left-hand column, last paragraph; figure 4</p> <p style="text-align: center;">--- -/-</p>	<p>1-32, 61-96, 121-157, 177-221, 255-267</p>

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- *&* document member of the same patent family

Date of the actual completion of the international search

15 May 1996

Date of mailing of the international search report

04.06.96

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Authorized officer

Fuhr, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/13231

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF INFECTIOUS DISEASES, vol. 167, no. 6, June 1993, pages 1474-1477, XP000569595 M. CAPELLO ET AL.: "Ancylostoma Factor Xa Inhibitor: Partial Purification and Its Identification as a Major Hookworm-Derived Anticoagulant In Vitro" see page 1475, left-hand column, line 3 see page 1476, left-hand column, paragraph 1 - page 1477, left-hand column, paragraph 1	1-32, 61-96, 121-157, 177-221, 255-267
P,A	WO,A,94 25000 (UNIV YALE ;CAPPELLO MICHAEL (US); HOTEZ PETER J (US); RICHARDS FRA) 10 November 1994 SEQ ID 8 see page 13, line 8 - page 19, line 5; claims	1-267
X	CHIMICAL ABSTRACTS, vol. 113, no. 18, 29 October 1990 Columbus, Ohio, US; abstract no. 158738, OKLAHOMA MEDICAL RESEARCH FOUNDATION, USA: "Thrombin-binding polypeptides as antithrombotic agent for artificial organs or other surfaces" XP002002737 see compound 129818-54-8 see abstract & JP,A,02 019 399 (OKLAHOMA MEDICAL RESEARCH FOUNDATION, USA)	1,31,61, 65,72, 73,97, 120,132, 134,138, 139,158, 177,181, 188,189, 222,255
X	WO,A,88 09811 (NOVO INDUSTRI AS) 15 December 1988 see claims; figures 7,8,11; examples	1,31,61, 65,72, 73,97, 120,132, 134,138, 139,158, 177,181, 188,189, 222,255
X	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 232, no. 1, July 1984, SAN DIEGO, US, pages 143-161, XP000570396 D.R. BABIN ET AL.: "The Isoinhibitors of Chymotrypsin/Elastase from Ascaris lumbricoides: The Primary Structure" see figures 4,10,11	1,31,61, 65,72, 73,97, 120,132, 134,138, 139,158, 177,181, 188,189, 222,255

INTERNATIONAL SEARCH REPORT

Inter- national Application No
PCT/US 95/13231

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EP,A,0 255 771 (INTEGRATED GENETICS INC) 10 February 1988</p> <p>see claims; examples -----</p>	<p>1,31,61, 65,72, 73,97, 120,132, 134,138, 139,158, 177,181, 188,189, 222,255</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/ 13231

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.: X
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information Sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 65-68, 125-128, 181-184 and 259-262 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/13231

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9425000	10-11-94	US-A- 5427937 EP-A- 0697857	27-06-95 28-02-96
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